THE EXPRESSION AND FUNCTION OF LCK IN CHRONIC LYMPHOCYTIC LEUKAEMIA

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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CONTENTS

ABSTRACT

B-cell antigen receptor (BCR) [engagement by antigen/signalling] contributes to Chronic lymphocytic leukaemia (CLL)-cell selection and survival. This signalling pathway has been investigated and the main Src family kinase (SFK) involved has been identified as Lyn, as in normal B cells. Expression of another SFK Lymphocyte specific tyrosine kinase (Lck) has also been described in CLL cells but the factors leading to the expression of this kinase, which is essential for T cell antigen receptor signalling, and its contribution to CLL cell survival have not been previously investigated. This study confirms the presence of Lck mRNA and protein in CLL cells as previously shown but uses highly purified cells to ensure that there was no contamination of T cell-specific Lck affecting the quantification as was thought to be the case in some B-1 cell studies. The levels of protein vary from 20 to 256pg per μg of total cellular protein and do not correlate with CLL prognostic markers or the amount of Lck mRNA. The regulation of the expression of the protein was investigated and suggested that the mechanism differs from that of T cells. It was shown that expression of Lck protein cannot be stimulated in CLL using IL-2 and crosslinking of the BCR, which can induce Lck protein in normal B cells. Incubation of CLL cells for 48 hours with an Lck inhibitor induced apoptosis in all CLL cases to some extent, but to a greater degree in unmutated (UM) than mutated cases ($p<0.01$). Stimulation-induced survival of CLL cells by CpG, anti-IgM or CD40 ligand was also reduced by the Lck inhibitor. The specificity of the Lck inhibitor for Lck and not other Src family kinases was confirmed by analysis of the activating tyrosine residues in Lck (Y394) and Lyn (Y396) following treatment of CLL cells with the inhibitor. The mechanism of Lck-induced survival was investigated and it was shown that treatment of CLL cells with the Lck inhibitor decreased phosphorylation of ERK, IKK and Akt which contribute to CLL-cell survival in response to BCR engagement. Pro and anti-apoptotic proteins were also investigated following treatment with the Lck inhibitor and cleavage of Bcl-xL and a reduction in Mcl-1 levels were observed. This study shows that Lck has a pro-survival role in CLL, especially in UM cases, and would therefore be a potential therapeutic target either with a specific targeted therapy or using a general Src kinase inhibitor such as Dasatinib which would also inhibit Lyn.

ACKNOWLEDGMENTS

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In particular I would like to thank Prof. Zuzel for allowing me the opportunity to undertake this study and for his advice, support in writing this Thesis and guidance throughout. I would also like to thank Dr. Slupsky for his immense support, especially in the final year.

DECLARATION

The work presented in this Thesis is my own, with the exception of practical assistance during the pSrc immunoprecipitation work which was provided by Dr. Slupsky.

LIST OF FIGURES

ABBREVIATIONS

µg. Microgram (10^{-6} grams)

µM. Micromolar (10^{-6} molar)

BCR. B cell antigen receptor

BSA. Bovine serum albumin

CD40L. CD40 ligand

CLL. Chronic Lymphocytic Leukaemia

CLP. Common lymphoid progenitor

CMP. Common myeloid progenitor

Csk. C-terminal Src kinase

DIABLO. Direct IAP binding protein with low pI

$DIOC_6$. 3,3-dihexyloxacarbocyanine iodide

DMSO. Dimethyl sulfoxide

DNA. Deoxyribonucleic acid

ECL. Enhanced chemi-luminescence

FACS. Fluorescence activated cell sorter

FCS. Fetal calf serum

FITC. Fluorescein isothiocyanate

GMP. Granulocyte-macrophage progenitor

HC. Hairy cell

HCL. Hairy Cell Leukaemia

HRP. Horseradish peroxidase

IAPs. Inhibitor of apoptosis proteins

IgM. Immunoglobulin M

IgVH. Immunoglobulin variable heavy chain

IL-2. Interleukin 2

ITAM. Immunoreceptor tyrosine based activation motif

LAC. Lactacystin

Lck. Lymphocyte specific tyrosine kinase

LMPP. Lymphoid multipotent progenitor cell

LPS. Lipopolysaccharide

MAPK. Mitogen activated protein kinase

MCL. Mantle cell lymphoma

MEK. Mitogen activated protein kinase- kinase

mRNA. Messenger Ribonucleic Acid

NFAT. Nuclear factor of activated T cells

NFκB. Nuclear factor κB

PAGE. Polyacrylamide gel electrophoresis

PBS. Phosphate buffered saline

PE. Phycoerythrin

(p)ERK. (phosphorylated) Extracellular signal regulated kinase

PI. Propidium iodide

PKC. Protein kinase C

PLC. Phospholipase C

PMA. Phorbol 12-myristate 13-acetate

PMSF. Phenylmethanesulfonyl fluoride

PP1. (4-amino-5-[4-methylphenyl]-7-[t-butyl]pyrazolo[3,4-d]pyrimidine)

RIPA. Radio-immunoprecipitation assay buffer

RT-PCR. Reverse transcription polymerase chain reaction

SAC.

SDS. Sodium dodecyl sulphate

SFK. Src family kinase

SH. Src homology domain

smac. Second mitochondrial activator of caspases

Src. Cellular homologue of transforming gene of Rous sarcoma virus

TAE. Tris-acetate-EDTA

TBST. Tris buffed saline with tween-20

TCR. T cell antigen receptor

TLR. Toll like receptor

WCL. Whole cell lysate

XIAP. X-linked IAP

XL. BCR crosslinking

Zvad. Z-Val-DL-Ap-fluoromethylketone

Chapter 1

General introduction

1.1 Overview

The subject of this thesis is the role of the Src-family kinase (SFK) Lymphocyte specific tyrosine kinase (Lck) in the pathogenesis of Chronic Lymphocytic Leukaemia (CLL). Lck was originally found to be expressed in T cells [1] where it plays a crucial role in their development and function [2]. The enzyme was later found to be also expressed in subsets of normal and malignant B cells, including CLL cells [3, 4]. The function of Lck has been clearly defined in T cells and its equivalent role in B cells has been mainly ascribed to another SFK Lyn [5, 6]. The contribution of Lck to the development and function of normal or malignant B cells remains to be defined.

1.2 Cell of origin in CLL

1.2.1 Haematopoietic cell differentiation

All classes of blood cells are derived from hematopoietic stem cells (HSCs) in adults, partly through progressive loss of differentiation potentials for other cell lineages. Once the HSC receives signals to undergo differentiation, there is a gradual loss of self-renewal ability. The

traditional view of the decision between the two lineages has been revised in recent years. New models of haematopoiesis have been formed due to the identification of progenitors with diverse cell fates [7-11].

In addition to transcription factors, increasing evidence suggests that microenvironments in the bone marrow may play a role in lymphoid specification and commitment.

Hematopoietic cells can be separated into two major lineages: the lymphoid lineage which includes T, B, and natural killer (NK) cells, and the myeloid lineage comprised of erythrocytes, megakaryocytes, granulocytes, and monocyte/macrophages [12].

1.2.2 Lymphoid versus myeloid cell fate selection

The identification of distinct lymphoid and myeloid progenitor cells [known as the common lymphoid progenitor (CLP) [13] or common myeloid progenitor [7] (CMP)] supported a model whereby the two lineages develop independently downstream of HSCs (figure 1.1a). However, more recent work using FACS analysis to identify different progenitor cell populations has challenged this traditional view, and allowed a new developmental pathway model to be formed as shown in figure 1.1b. Both models support differentiation from CD34 positive multipotent progenitor cells (MPPs). The model in figure 1.1b is now

favoured because FACS analysis of CD34 positive MPPs showed that this population is far more heterogeneous in terms of antigen expression than was originally thought.

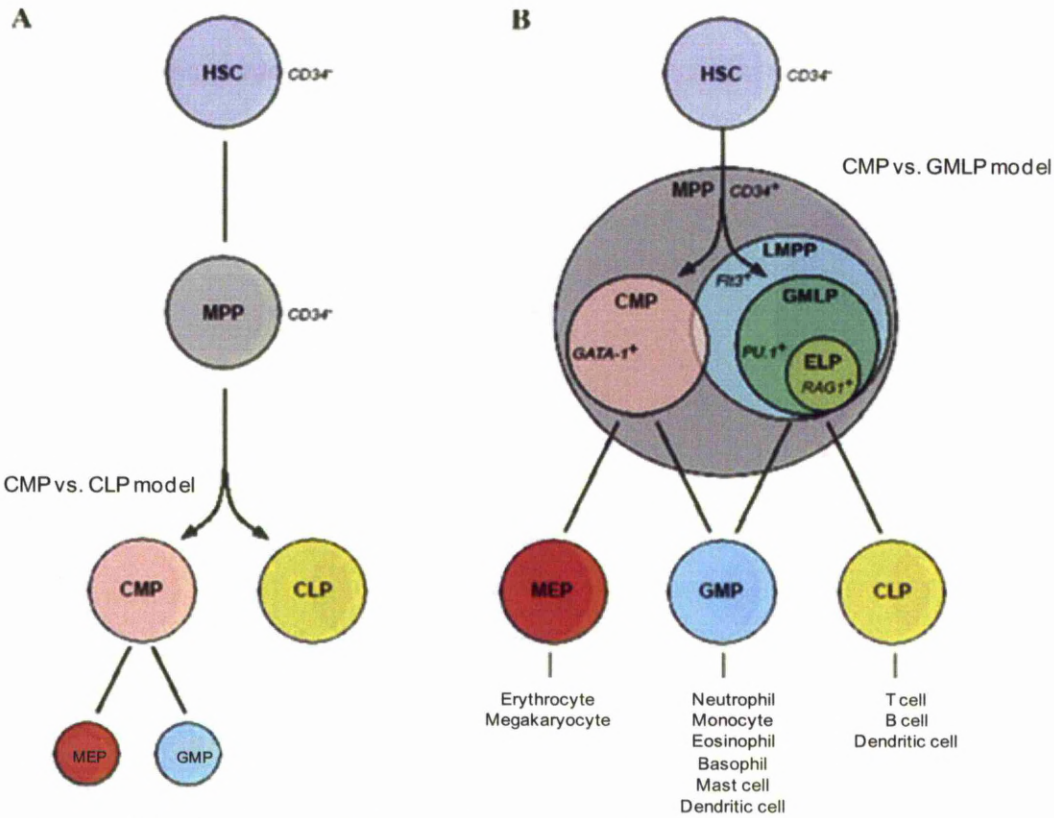


Figure 1.1 Summary of the current model of lymphoid versus myeloid fate decision [14].

Lineage commitment to CMPs appears to be dependent upon expression of the transcription factor GATA-1. With respect to differentiation to the CLP a series of steps seems to be required; the first of these steps involves

expression of Flt3 which appears to direct cells to differentiate into the LMPP (lymphoid multipotent progenitor cells). The second step involves expression of the transcription factor PU-1 and this assists the differentiation of cells into the granulocyte-macrophage progenitors (GMP). The final step of differentiation to CLP requires the expression of the Ikaros gene. The CLP gives rise to either T, B or lymphoid dendritic cells.

1.2.3 B cell differentiation

Commitment of CLPs to the B lineage is controlled by E2A family transcription factors, and expression of the components that are required for antigen receptor gene rearrangement (RAG/TdT genes). Expression of the IgH and L chain genes and the mb-1 gene responsible for production of Igα (part of the B cell receptor complex) are seen as signs of irreversible differentiation.

B lymphocytes express clonally diverse cell surface immunoglobulin (Ig) recognising specific antigenic epitopes. B cell development begins in the bone marrow and then finishes in the secondary peripheral lymphoid tissue [15]. Within the bone marrow pro B cells differentiate to pre B cells and then to immature B cells (figure 1.2). Rearrangement of Ig genes causes surface expression of a pre-B cell receptor composed of Igμ

heavy and surrogate light chains. The presence of this receptor is necessary for both further differentiation of the cell as well as clonal selection. Rearrangement of variable, diversity and joining (VDJ) gene segments occurs during the transition of pre B cells to immature B cells which express the final mature BCR structure on their surface. VDJ recombination is necessary to give antigen binding diversity to the developing antibody expressing clones. At this point in development, B cells must pass a checkpoint that ensures self reactive cells do not develop further because 75% of human early immature B cells are self-reactive [16]. Editing of the BCR [17, 18] and deletion of self-reactive clones [19] are involved in this selection process. If a cell passes this selection checkpoint then it is able to exit the bone marrow as a transitional B cell. These cells can then eventually progress into mature follicular B cells or marginal-zone B cells. Further development can also occur during an immune response when antigen-specific B cells differentiate into either plasma cells capable of secreting antibodies or memory B cells. This maturation and selection procedure is summarised in the following diagram:

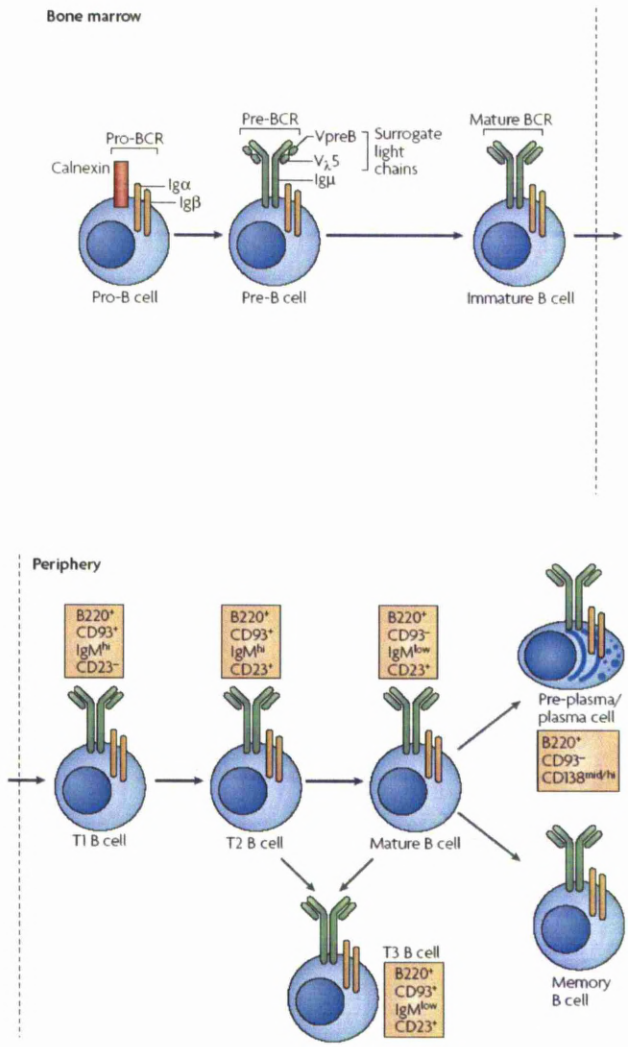


Figure 1.2. Summary of the development of B cells taken from [20]. Naive cells in the bone marrow enter the periphery and can then undergo maturation upon encounter with antigen.

The early stages of B cell development are regulated by at least 10 transcription factors (TFs) [15]. E2A, EBF and Pax5 are the main TFs responsible for B-lineage commitment and differentiation. Pax-5 deficient pro-B cells have the capacity to adapt non-B-lineage fates and can develop

into other haematopoietic lineages due to the role of Pax5 in activation of genes responsible for B-cell development and repression of genes involved in non-B lineage development [21].

1.2.4. The cell of origin in CLL

The cells which give rise to CLL have not been identified. However, certain assumptions can be made about the B cell population that is likely to support the genesis of CLL. During differentiation B cells are either negatively or positively selected through interaction with cognate antigens. Negative selection takes place at an earlier stage of maturation and involves cells undergoing apoptosis due to high-affinity interaction with self antigens [22, 23]. However, some cells can be positively selected by reaction with low affinity self antigens at low concentrations. These cells give rise to a distinct lineage of CD5 positive B cells known as B-1 cells [24]. The remaining surviving cells give rise to B2 cells with specificity for exogenous antigens of microbial or other origin [25].

The currently held view is that B-1 cells found at locations such as peritoneum and marginal zone of the spleen are the most likely cells of origin of CLL. This view is supported by gene expression profiling studies which show that the malignant cells have a common genotype that resembles normal mature memory B cells [26]. The B-1 origin is

further supported by the CD5 positivity of CLL cells, and by their specificity for low affinity self antigens and structurally similar antigens of microbial origin [27]. As I have discussed above, the gene segment usage that is used in CLL cells shows that the spectrum of these antigens is relatively narrow and that a substantial majority of CLL cells share specificity for the same antigens [28, 29]. This excludes a random usage of these segments and favours the proposition that CLL cells have been positively selected by their cognate antigens.

CLL cell maturation frequently involves interaction with T-independent antigens of the type known to be responsible for development of B-1 cells. At least in mouse, the signalling generated by these antigens requires expression of Lck [30].

1.3. Chronic Lymphocytic Leukaemia

Leukaemias are cancers of the cellular component of blood, and can arise from any of these components (i.e. lymphocytic or myelogenic) at potentially any stage of their differentiation. Leukaemias can be divided into either acute or chronic forms based on the speed of disease progression. CLL is a leukaemia of mature B lymphocytes that is defined by a slow accumulation of malignant cells in the blood, bone marrow and lymphoid tissues.

CLL is a prevalent disease that typically affects people over the age of 60, and affects men more than women [31]. CLL is characterised by a clonal expansion of mature malignant CD5, CD19 and CD23 positive B cells that have reduced membrane levels of IgM, IgD and CD79b [32, 33]. Other molecular markers and gene expression profiles demonstrate that CLL cells have a phenotype [34] and genotype [26] of mature activated memory B cells. Another characteristic of CLL cells is that they are locked into the G0 stage of the cell cycle and are largely unresponsive to mitogenic stimulation [35].

The clonal expansion of CLL cells was originally attributed primarily to prolonged *in vivo* survival of these cells due to a defect in apoptosis [36]. However, the use of deuterium labelling has recently been used to demonstrate an equal importance of cell proliferation to the clonal expansion of CLL cells. In this study it was suggested that the balance between proliferation in growth centres and cell death outside of these centres governs progression of the disease. Thus, a combination of proliferation and resistance to apoptosis is likely to be responsible for disease progression. This notion is supported by a further study showing that CLL cell proliferation is reduced when compared to that of normal B cells [37]. Despite a considerable amount of research into the pathobiology of CLL, the exact events leading to oncogenic transformation in the malignant cells remain uncharacterised.

CLL is a disease that can progress differently in different patients; some patients surviving for many years without the need for therapy, whilst others have a fatal disease that progresses rapidly despite treatment. This heterogeneity in progression was defined by the Rai [38] and Binet [39] staging systems which are the basis for prognosis assessment. In these systems patients are classified according to different parameters. These parameters include white blood cell count, cell doubling time, and infiltration of bone marrow and secondary lymphoid organs resulting in the suppression of function. Rai stage 0 and Binet stage A patients have an average predicted survival of 15 years. Intermediate cases are Rai stages I or II and Binet stage B; these cases have a median survival of 5 to 7 years. High risk patients are classified as Rai stage III or IV and Binet stage C and survive on average for less than 3 to 4 years [40].

Prognosis of the potential for CLL to progress is evident also from recently identified molecular and immunophenotypic traits. The traits that are the most powerful predictors of disease progression include mutation of the variable region encoded by the gene for the immunoglobulin heavy chain (IgVH), expression of proteins such as CD38 and ZAP70 [41] and the presence of certain cytogenetic abnormalities involving chromosomes 13q14, 11q23, 17p13, 6q21 and 12q13 [42]. Currently, decisions on disease prognosis are made on the basis of both the Rai and Binet clinical staging criteria plus the molecular markers listed above [41].

I will now discuss how these different molecular markers of disease prognosis contribute to the pathobiology of CLL.

1.4.1 Factors proposed to play a role in the pathogenesis of CLL

1.4.1.1 The role of the B cell antigen receptor

The usefulness of determining IgVH mutation status in CLL disease prognosis was first described in 1999 [43, 44], and has since been corroborated several times. These studies defined CLL cases as having unmutated IgVH genes if the VH sequence within the malignant cells did not differ by more than 2% from the germ-line sequence [44]. Thus CLL cases which have less than 2% somatic mutation have a poor prognosis when compared to cases where the mutation is greater than 2%.

Classically, somatic mutation of IgVH genes occurs within germinal centres at the rate of 10^{-3} to 10^{-4} mutations per base pair per generation [45, 46] and involves the presentation of antigens by B cells to cognate helper T cells which then provide soluble and cell-contact stimuli which assist B cell proliferation and survival [47] (so called T-dependent antigen response). Based on the role of the germinal centre in providing the environment for somatic mutation to take place it was originally thought that CLL might comprise 2 diseases; one which arose from cells which had unmutated IgVH genes and likely had not entered the germinal centre, and

one which had mutated IgVH genes and therefore had passed through the germinal centre [48].

However, it has been noted that under some circumstances, in normal B cells, IgVH mutation can take place outside germinal centres and in the absence of T cells [49-51]. This model probably reflects CLL because analysis of global gene expression has shown that CLL cases bearing mutated and unmutated IgVH genes are largely similar to each other in their gene expression profiles, and that this likely results from a common cell of origin rather than from cells that are at different stages of differentiation.

Analysis of IgVH mutational status in CLL also revealed that the malignant cells use a characteristic repertoire of Ig genes in the final assembly of the antigen receptor expressed on the cell surface. These genes code for the VDJ segments of the antigen binding domain of Ig and give diversity to the antigen binding characteristics of the antibody through recombination. The biased usage of particular VDJ genes in CLL has led to the hypothesis that antigen binding to the BCR is an important component of CLL pathogenesis. For example, cells that utilise the VH3-21 gene segment for recognition of antigen have a particularly unfavourable disease prognosis likely due to the prevalence of this autoantigen in the body [27]. The difference that IgVH mutation makes in determining disease prognosis

is likely played out in antigen specificity of the BCR expressed on the malignant cells of CLL. Hervé et al [52] recently showed that antibodies derived from unmutated CLL were polyreactive whereas those derived from mutated CLL were not. Furthermore, this same study also showed that mutation of the IgVH sequence within antibodies derived from mutated CLL back to the germline sequence restored polyreactivity of these antibodies. Based on this evidence it is easy to hypothesise a role for BCR engagement in the pathogenesis of CLL.

Thus, preferential positive selection and expansion of CLL-cell clones likely involves alterations in BCR signalling in comparison with the cells that are negatively selected through interaction with antigen. Generally, BCR signalling in CLL cells is strongly attenuated in comparison with signalling in normal B cells. This could be the consequence of chronic antigenic stimulation resulting in different degrees of cell anergy, and to specific changes in gene expression profiles favouring clonal expansion of these cells [53]. Chronic stimulation by antigen is a consequence of the arrest of CLL cells at a pre-plasma-cell stage of maturation resulting in the failure of the cells to fully differentiate *in vivo* and secrete significant amounts of antibody required for the clearance of the antigen. Although these chronically stimulated cells can be anergised with respect to some responses such as Akt activation, the chronic stimulation may maintain activity of several other signalling pathways required for cell survival [53].

Thus, such anergised CLL cells contain constitutively phosphorylated MEK 1/2, ERK 1/2 and IκBα together with an increase in activated NF-AT [53].

In addition to the role of the BCR, CLL cases with unmutated IgVH genes are likely to have additional features that contribute to unfavourable prognosis. Some insight into these additional features is provided by frequent co-expression of ZAP-70 and CD38 in these cells which may provide the signals for their accelerated clonal expansion [54].

1.4.1.2 The role of CD38

CD38 is a 42kDa type II transmembrane glycoprotein that is expressed on various haematopoietic cells, including normal B and T cells [55], and also on non-haematopoietic cells [56, 57]. CLL cases where expression of CD38 can be detected on 30% or more of the malignant cells, and where these same cells contain IgVH sequences that retain germline or close to germline configuration typically have a poor disease prognosis [44, 58]. This is because CD38 expression is associated with CLL cells which are more rapidly proliferating [54, 59]. The exact role of CD38 in CLL pathogenesis is unclear, but may be due to the capacity of this protein to catalyse ADP ribosylation and modulate intracellular calcium levels [56].

1.4.1.3 The role of ZAP70

ZAP70 is a non-receptor tyrosine kinase strongly expressed in T cells and NK cells [60]. This protein is either absent or expressed at a very low levels in normal B cells [61] but the expression of this protein can be upregulated in B cells undergoing the germinal centre reaction [62]. The presence of ZAP70 in CLL cells is an indicator of unfavourable prognosis [63] and is strongly correlated with cases where the IgVH genes remain unmutated. Measurements of the expression of this protein in the malignant cells of CLL is sometimes used as a simple surrogate for the more costly and time-consuming measurements of IgVH mutation [64, 65]. The role of ZAP70 in BCR signalling will be discussed below.

1.4.1.4 Cytogenetic abnormalities

In addition to the use of the genotypic and phenotypic attributes listed above for disease prognosis in CLL, the presence of particular chromosomal aberrations within the regions 13q14, 11q23, 17p13, 6q21 and 12q13 can also be used. Deletion of 11q23 and 17p13 are associated with aggressive CLL because the genes that are affected are ATM and p53. The proteins that are encoded by these genes are responsible for regulating cell response to DNA damage, therefore, drugs which affect DNA, such as the nucleoside fludarabine^{TM} which is commonly used to treat CLL, are ineffective. On the other hand, deletion of 13q14 is a

favourable prognostic indicator possibly due to its effect on the expression of microRNAs such as miR-16-1 and miR-15-a [66].

1.4 Intracellular signalling in B cells.

T and B cell development and function are principally regulated by signals generated through interaction of their respective antigen receptors (TCR and BCR) with antigen [67, 68]. Antigen receptor-generated signals have been shown to be initiated through activation of Lck in T cells [69] and Lyn in B cells [5]. These signals then branch into a number of pathways which regulate selection, maturation, function and survival of the cells.

1.4.1 Src Family Kinases

There are 8 different SFKs expressed in human cells, these are; Lck [1], lyn [70] fyn [71], src [72], yes [73], fgr [74], hck [75] and blk [76]. These enzymes are non-receptor protein-tyrosine kinases that have been implicated in regulation of metabolism, survival, proliferation, differentiation and migration of cells belonging to different lineages [77, 78]. SFKs range in size from 52 to 62 kDa and all are composed of eight functional regions. The eight regions are: a myristoylated site, a unique region, a src homology (SH) 4 domain, an SH3 domain, an SH2 domain, a linker region, a kinase/catalytic domain and a regulatory domain [79]. The basic structure of SFKs is shown in figure 1.3.

Figure 1.3. The structure of Src family kinases.

The regions most relevant for activation of SFK enzymes and for their interaction with substrates are the SH2 and SH3 domains. These regions form the regulatory domain of SFKs and maintain a closed inactive conformation through intra-molecular protein-protein interactions when the kinase is at rest. These motifs also regulate substrate specificity by mediating inter-protein interactions. The SH2 domain is ~100 amino acids long and interacts with phosphotyrosine motifs of substrate proteins [80] as well as with the inhibitory phosphotyrosine site on the kinase itself. The SH3 domain is ~50 amino acids long and binds to proteins that contain proline rich motifs related to the PXXP consensus [81, 82].

An additional important feature of all SFKs is the presence of conserved inhibitory and activating tyrosine residues. The inhibitory tyrosine residue is contained within the C terminus of the SFK and, when phosphorylated, maintains the protein in a restrained inactive conformation through interaction with the SH2 domain of the protein. The activating residue is located within the kinase domain and, when phosphorylated, facilitates enzymatic activity.

1.4.2 Unique region of Src family kinases

SFKs have a similar domain arrangement, but the N-terminal region of all family members is the most divergent and has been termed the unique domain. This domain directs fatty acid acylation which occurs as the protein is translated [77]. N-terminal myristoylation is a permanent modification that directs SFKs to the cholesterol-rich membrane rafts [83], whereas palmitoylation of SFKs within this region is reversible [84, 85]. The unique domain of SFKs also provides additional interaction and phosphorylation sites that are thought to be involved in the specific targeting and regulation of each enzyme. For example, in T cells the unique domain of Lck has been shown to be associated with the cytoplasmic tails of CD4 and CD8 [86]. For the purposes of this thesis we will concern ourselves only with Lck.

1.5 Function and expression of Lck

1.5.1 The role of phosphorylation in regulating Lck function

As with other SFKs, phosphorylation of Lck plays an important role in its function. Activation of Lck in T cells involves CD45-dependent dephosphorylation of the inhibitory phosphotyrosine (Y505). This dephosphorylation results in a structural change/unfolding of the protein that is followed by autophosphorylation in the active site (Y394). The unfolding of the protein also enables the binding of substrates and other molecules (figure 1.4a) [87].

The refolding of Lck into its deactivated form occurs via dephosphorylation of Y394 and rephosphorylation of Y505. Dephosphorylation of the activating phosphotyrosine in Lck is suggested to be mediated by CD45 [88-90], PEP (proline-, glutamic acid-, serine-, and threonine-rich (PEST) domain-phosphatase) [91], protein tyrosine phosphatase (PTP)-PEST [92], and SH2 domain-containing phosphatase 1 (SHP-1) [93]. Rephosphorylation of the inhibitory tyrosine in Lck is mediated by C-terminal src kinase (Csk) [94] (figure 1.4b). As mentioned above, the phosphorylated Y505 binds to the SH2 domain of Lck, blocking substrate access to the enzyme. In addition, the SH3 domain associates with the proline rich region within the linker domain of the protein [82]. The culmination of these two interactions is to force the catalytic domain into a state where the residues responsible for phosphorylation are no longer in their optimal configuration [95]. This is illustrated in figure 1.4.

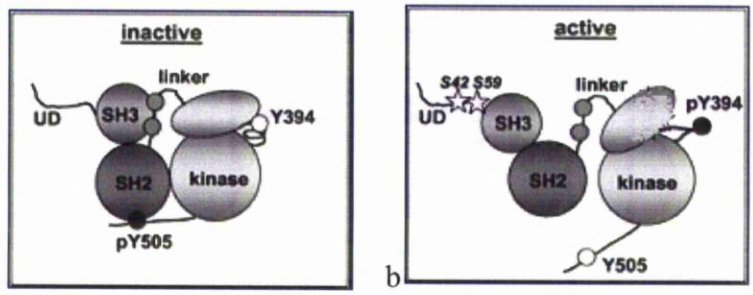


Figure 1.4. Activation and deactivation of Lck (modified from[96]).

Active Lck can be further phosphorylated on serine residues by protein kinase C (PKC) and by extracellular regulated kinase (ERK). PKC and ERK catalyse the phosphorylation of serine 42 and 59 within the unique region of the protein [97, 98]. This results in a shift in the apparent molecular weight of the protein from 56kDa to 60kDa when analysed by SDS-PAGE [99-101]. This appearance of the 60kDa form is taken as proof that Lck has been serine phosphorylated on least one of the serine 42 or 59 sites [102, 103]. The function of serine phosphorylation is postulated to regulate the ability of Lck to interact with protein tyrosine phosphatases such as SHP-1, and therefore act in a positive feedback fashion to stabilise the activity of Lck [104, 105]. Serine phosphorylation of Lck is also thought to mark the protein for ubiquitination and destruction via proteasomal degradation [96] (figure 1.4.b).

1.5.2 Tissue expression of Lck

Lck is primarily expressed in cells of haematopoietic origin, and, in particular, in T and NK cells [106]. However, Lck can also be found in a variety of other normal and malignant cells. In normal cells, the expression can be cell-maturation or activation dependent. For example, human and murine B-2 cells express Lck as immature cells, but not as quiescent mature cells [107]. However, stimulation of B cells in the germinal centre has been reported to induce the expression of this protein in these cells [108]. Additionally, Lck has been found in mouse peritoneal CD5+ B-1a

cells, but not in the CD5+ B-1 cells located in the marginal zone of the spleen. This difference is reported to depend upon different specific microenvironmental stimuli at the two locations [109].

With respect to Lck expression in malignant cells, Lck is found in tumours of the breast [110], lung [111] and colon. Lck is also expressed in B cell malignancies such as DLBCL, mantle cell lymphoma and B-ALL [108, 112].

1.5.3 Transcriptional regulation of Lck

The expression of Lck during T and B cell development and activation by stimuli such as antigen-receptor activation and IL-2 is primarily regulated by transcription [113]. The human Lck gene is located on chromosome 1q35-34.3 and has 12 exons distributed across ~14kb of genomic DNA. There are 2 promoters that are responsible for regulating expression of the gene, a proximal and a distal promoter. This leads to 2 main types of transcripts [114] which have been further diversified based on length [115]. Activation of the proximal, type I, promoter occurs in T-cell leukaemia cell lines and non-lymphoid neoplasms such as colon carcinoma [111]. Activation of this promoter is thought to originate from the absence of a translational repressor [116].

Activation of the distal, type II, promoter which is 35 kilobases upstream of the coding region of the Lck gene, occurs in mature peripheral T cells and at low levels in myeloid cell lines [115]. Malignant lymphoid cells have been shown to contain both transcripts [115].

1.5.4 Lck signalling

The most extensively studied role of Lck is in T cells where this enzyme is involved in cell activation and IL2 production in response to TCR engagement. In these cells, Lck initiates signalling via phosphorylation of immuno-receptor tyrosine activation motifs (ITAMS) [117] within CD3 and the TCRζ chain which allows recruitment of zeta-associated protein kinase of 70kDa (ZAP70) to the TCR [118]. ITAM-bound ZAP70 is then tyrosine phosphorylated by Lck [119] allowing ZAP70 to phosphorylate substrates such as SLP76 and LAT leading to activation of the subsequent steps of the TCR signalling cascade [120]. This is summarised in figure 1.5.

In response to certain stimuli, active Lck also associates with CD38 and leads to endocytosis of this protein. This is thought to result in the production of cyclic ADP ribose and subsequent increase in intracellular calcium levels leading to NF-AT activation [121].

In addition to its role in T cell activation, Lck also plays a role in the

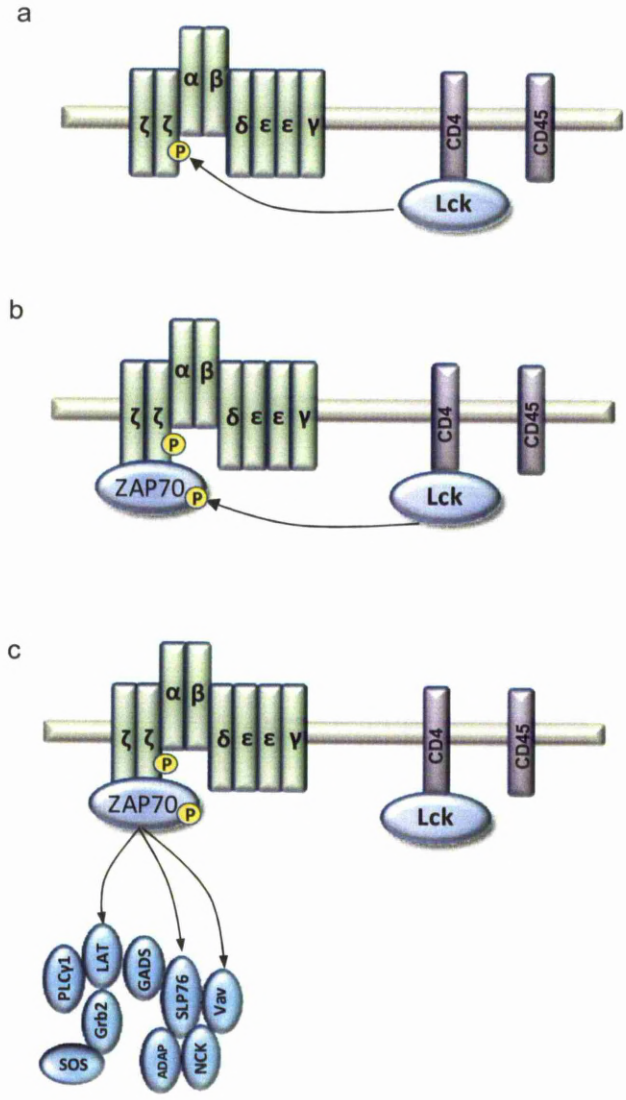


Figure 1.5. The role of Lck in TCR signalling.

induction of tolerance upon protracted TCR signalling. The induction of tolerance by Lck could involve phosphorylation and recruitment of CD5 [122, 123] and c-Cbl to the TCR signalosome. In this model CD5 contributes to tolerance by recruiting SHP-1 phosphatase to oppose the action of protein tyrosine kinases [123], including Lck. c-Cbl, on the other hand, contributes to tolerance via its ubiquitin ligase activity, leading to ubiquitination and proteasomal degradation of several signalling components including Lck itself [124, 125].

Although it has been demonstrated that Lck is expressed in B cells, its role within these cells is not clear. One report has indicated that Lck expression confers hyporesponsiveness to B-1 cells undergoing BCR stimulation in the peritoneum [109]. However, these findings are contrasted by another study reporting that Lck is necessary for sustained BCR signalling in these same cells [126]. Meanwhile, a third study has indicated that hyporesponsiveness of B-1 cells to BCR stimulation has nothing to do with Lck expression at all [127]. Thus, there is a lack of consistent evidence to support a direct role for Lck in BCR signalling.

1.5.5 The presence of Lck in CLL cells

In 1991, Abts *et al* [128] investigated the expression of Lck mRNA in 21 CLL samples using northern blot analysis and found that all samples contained transcripts for this gene. Normal B cells from the peripheral

blood of healthy donors were also investigated in this study but these did not contain any Lck transcripts. Lck protein expression in CLL cells was later confirmed in subsequent studies by western blot analysis [4, 129-131]. Some of these studies also used protein kinase assays to confirm the presence of active Lck in CLL cells, and to further show that stimulation of these cells with SAC/anti-IgM caused a change in the expression level and the formation of a higher molecular weight species of Lck. However, some doubt has been expressed about the ability of CLL cells to express Lck [127, 132].

1.6. Lck in B-1 cells

B-1 cells were originally distinguished from conventional B cells (B2) due to the expression of CD5 [133], a glycoprotein expressed on the surface of T cells that is likely to regulate signals emanating from the TCR. B-1 cell development is antigen driven and potentially reflects selection by thymus-independent self antigens [109]. Maintenance of the B-1 population appears to be also dependent on BCR signals, because this population is affected when mutations are present in proteins that participate in signalling via the BCR [134]. Further work on B-1 cells has revealed that there are two subpopulations within this group; B-1a cells which are characterised by their expression of CD5, and B-1b cells which do not express this antigen [135, 136].

Another characteristic feature of B-1 cells is Lck expression. A role for Lck in B-1 cell physiology is implicated in a publication by Ulivieri *et al* [126]. Here it was shown that B-1 cells developed normally in $Lck^{-/-}$ mice, but that the BCR signalling in these cells was defective. This shows that Lck is not required for B-1 cell development, but required for effective BCR-induced signal transduction in these cells.

Alternatively, Lck may be required to confer the BCR hyporesponsiveness seen in CD5+ B-1 cells. In this way, Lck appears to be involved in the maintenance of the unique function of these cells [109]. Chronic, low-level BCR stimulation, such as encounter with ubiquitous self-antigen is sufficient to induce CD5 expression [137, 138]. Lck expression however appears to require additional cues or signals which may be provided by environmental factors. Dal Porto *et al* [109] hypothesise that continued antigen exposure induces CD5 expression, regardless of the locale of the cells, but the peritoneum and pleural cavity provides a unique microenvironment in which prolonged residency causes phenotypic change of the B-1 cells such as Lck expression. Splenic B-1 cells do not express Lck; however upon entry to the peritoneum they can be induced to express this enzyme. In Lck deficient mice Dal Porto *et al* [109] showed that B-1 cell development is normal but peritoneal CD5+ B-1 cells are no longer hyporesponsive.

1.7 BCR signalling

As discussed above, Lck is described to play a role in either facilitating or downregulating BCR signalling. To understand any potential role of Lck in BCR signalling we must first understand how the BCR transfers its signal in normal B cells.

Engagement of the BCR induces receptor oligomerisation and phosphorylation of a non-covalently associated signalling unit, the Igα/β heterodimer (otherwise known as CD79α/β heterodimer). These proteins contain ITAMs, and are the target of the SFK Lyn [139]. Phospho-Igα/β are then able to recruit and activate Syk [140] and phosphatidylinositol 3 kinase (PI3K) either through direct interaction with their SH2 domains, or through interaction with adaptor proteins such as BCAP [141]. Activated PI3K generates phosphatidylinositol trisphosphate (PIP_3), which then recruits and activates Bruton's tyrosine kinase (Btk) [142, 143] as well as PDK1 and Akt [144-146]. In parallel, Syk phosphorylates the adaptor protein BLNK, which then acts as a scaffold to facilitate the phosphorylation of PLCγ2 [147] by Btk and Syk [148, 149]. Activated PLCγ2 hydrolyses PIP_2 to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates release of intracellular Ca^{2+}, which then together with DAG activates PKCβ. The latter kinase stimulates the NFκB pathway by interacting with the CARMA1–Bcl10–MALT1 complex to ultimately

activate IκB kinases (IKKs) [150, 151], as well as the JNK pathway [152]. DAG has an additional function in activating RasGRPs and lead to activation of the ERK pathway [153]. This pathway is illustrated in figure 1.6.

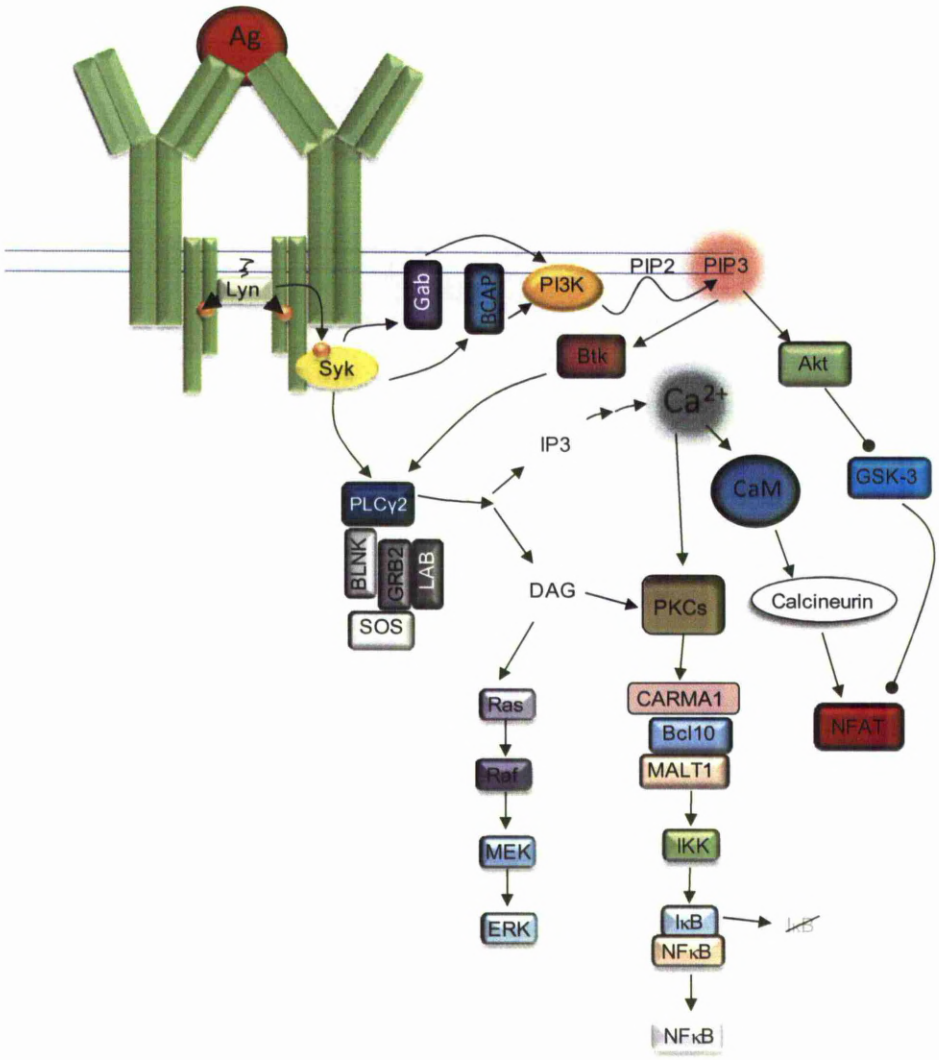


Figure 1.6. B cell antigen receptor-induced signalling in normal B cells.

Variation in this pathway has been noted between normal and anergic B cells [20]. For example, activation of the NFκB, Akt and JNK pathways have become uncoupled from the BCR in some models of B cell anergy [154-159]. Such uncoupling is thought to be mediated by chronic activation of SHIP-1, Dok and PKCδ [20].

Anergy seems also to play a role in BCR signalling in CLL cells. BCR crosslinking has been shown to stimulate the signalling pathways leading to activation of ERK, Akt and NFκB, but not the JNK pathway [160]. Moreover, in CLL cases expressing highly mutated IgVH sequences signalling to all pathways including the release of intracellular calcium is attenuated [161].

1.8. The potential role of Lck in CLL

The cellular components that have been proposed to play a role in attenuated BCR signalling in CLL cells include changes in expression or function of enzymes and adaptors which together form the BCR signalosome. These components include among others CD5 [162], c-Cbl [163] and Lck. Lck may play a role in the altered BCR signalling of CLL cells, possibly through its association with CD5 as has been proposed for peritoneal B-1 cells in mice, and in T cells. Porto *et al* [109] found that, in mice, Lck was required for the hyporesponsiveness of the BCR seen in

CD5+ B-1 cells. In T cells, Lck has been shown to be necessary for CD5 Y429 and Y463 phosphorylation [164] which is responsible for attracting SHP1 and downregulating TCR signalling. Considering that CD5 is expressed on CLL cells, it is therefore possible that Lck might perform a similar function. This is supported by studies showing that CD5 can be upregulated on conventional B cells that have been exposed to chronic antigenic stimulation *in vivo,* or by *in vitro* stimulation with anti-IgM and a soluble CD40 ligand [165]. CD5 expression on these cells exerts a negative feedback on BCR signalling as shown by reduced proliferative capacity and inhibition of intracellular calcium release following further BCR stimulation [166].

Renaudineau *et al* [167] showed that stimulation of CD5 with specific antibodies resulted in apoptosis of the malignant cells from a subset of CLL cases. The signalling in the cases that underwent apoptosis involved association of CD5 with the CD79a/CD79b components of BCR and the translocation of this complex to lipid rafts.

Lck may contribute to the disease progression of CLL through its role in positive signalling from proteins such as CD38 and ZAP70. These proteins are markers of poor prognosis in CLL; this might be particularly important with respect to ZAP70 expression in CLL cells because this protein is a major substrate of Lck in T cells [119]. Whether this could lead to further progression of signalling in CLL cells remains to be seen. However,

although the function of ZAP70 in CLL has not been clearly defined, it is known that this molecule facilitates BCR signalling [168].

Lck participation in cell stimulation may also involve interaction with CD38. As stated above, CD38 is a prognostic marker in CLL where it is routinely measured using FACS analysis. CD38 and Lck are physically associated in T cells and CD38 ligation increases tyrosine kinase activity of the CD38-associated Lck leading to activation of interleukin-2 gene transcription [169]. Figure 1.7 shows a summary of the proposed role of Lck in CLL cell survival.

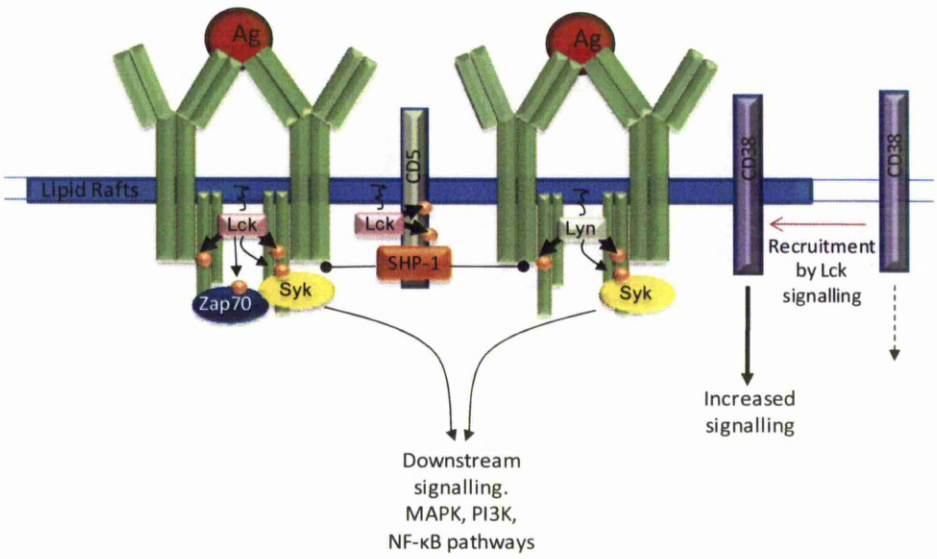


Figure 1.7. The theoretical role of Lck in CLL based on its function in T cells and B-1 cells.

1.9. Overall aims of the thesis

At the beginning of these studies expression of Lck in CLL cells had not been fully characterised. Therefore, the first aim of this work was to establish Lck expression in populations of purified CLL cells. Having shown that Lck is indeed expressed by CLL cells, I then sought to investigate how the expression of this protein is regulated. The second aim of this work was to establish a role for Lck in CLL pathophysiology. Thus, in the first instance I sought to establish a role for Lck is protecting CLL cells from spontaneous apoptosis. Secondly, I investigated a role for Lck in BCR signalling in CLL cells.

Chapter 2

Expression of Lck in CLL cells

2.1 Introduction

Work in this laboratory and the work of others [4] showed the presence of Lck protein in CLL cells. However, this was questioned in some other studies [132] suggesting that Lck is either not expressed in all CLL-cell clones, or that the expression is highly variable and depends upon stimuli to which some cells are not exposed.

CLL cells are thought to originate from B-1 cells and, in mice, only peritoneal B-1 cells, but not splenic B-1 cells, express Lck [30]. As Lck expression can be induced in normal B cells by antigen receptor stimulation [113] there is a possibility that the Lck-expressing CLL-cell clones develop under the influence of specific antigens and possibly also other stimuli that are present in the peritoneum but not in the marginal zone of the spleen.

Recently Lck expression was also found in germinal centre B cells [108] where it is induced by antigen and has an anti-apoptotic role. However, when these germinal centre cells give rise to plasma or memory cells, Lck

expression is down regulated since in the absence of stimulation these last two mature B cell types do not express this enzyme.

If it is assumed that Lck does play a non-redundant role in a particular subgroup of CLL, it would be important to establish how variable is its expression in different CLL-cell clones, how this expression is induced and what the contributions of gene transcription, translation and post translational modification are in the regulation of its expression.

An insight into the mechanisms involved in Lck expression in CLL cells can be obtained from the type of Lck transcripts present. These transcripts are produced from distinct promoters which are activated either during cell maturation or cell stimulation by some not yet fully identified stimuli [115]. The level of the Lck protein can also be primarily regulated by mRNA translation or by post-translational modification involving activation-dependent proteasomal degradation [96].

Lck mRNA production can be initiated from 2 separate promoters that result in the heterogeneity of the transcripts regarding the length and sequence of the 5' untranslated region (UTR). The actual coding region is however the same between the two types of the transcript which means that there is no functional difference between the translated proteins. Since the stimuli which initiate translation from the two promoters are different, it is

important to establish which transcript(s) are present in CLL, as this may identify the mechanism(s) that lead to the production of Lck in the malignant cells of this disease.

The type of transcript present in different cells depends on both cell type and stimuli to which a particular cell is exposed. Thus, the activation of the type I (proximal) promoter has been found in T cell leukaemia lines as well as in non-lymphoid neoplasms such as colon carcinoma. The type Ib transcript resulting from the activation of this promoter is present at a low level in B cell lines and is increased in malignancies of haematopoietic immature cells, such as AML [115].

The type II (distal) promoter is located 35 kilobases upstream of the coding region. Expression of the type II transcript is linked to the phenotype of activated cells and is upregulated during developmental transitions associated with antigen receptor signalling in both B and T cells [3]. This transcript is also present in presumably stimulated, mature, peripheral T and B cells as well as at the low levels in myeloid cell lines. The type IIa form (figure 2.1) is the major form of Lck mRNA present in human T cells. Lymphoid cells of a malignant origin contain both type I and II transcripts [170].

The aim of this chapter was to determine factors that regulate Lck expression at the level of mRNA, including usage of different promoters and the importance of translation in determining the level of Lck protein in CLL cells. Since it is well established that the level of Lck is also affected by post-translational modifications [96] this chapter also includes experiments in which Lck is activated either through BCR stimulation or PKC activation, followed by Western blot analysis of changes in electrophoretic mobility and amount of Lck protein caused by phosphorylation and proteasomal degradation.

2.2 Materials and methods

2.2.1 Cell isolation and culture

Peripheral blood samples were obtained from patients previously diagnosed with CLL, Hairy Cell Leukaemia (HCL) or Mantle Cell Lymphoma (MCL) after they gave informed consent and with the approval of the Liverpool Research Ethics Committee. The clinical and biochemical characteristics of all the samples used in this study are summarised in Table 1.2. Data for ZAP70 and CD38 were obtained by FACS while IgVH gene sequencing data was obtained as detailed before [171] and the information then stored on the departmental database. The majority of CLL cases used in this study had White blood cell counts (WBC) higher than $50x10^9$/L to limit the influence of non-CLL cells, specifically T cells which contain high levels of Lck protein. In experiments where purity of over 90% CLL cells was required, such as PCR and quantification of CLL-specific Lck protein, samples were negatively purified as detailed in section 2.2.4.

Mononuclear cells were obtained by centrifugation (500rcf, 30 minutes, room temperature) on Lymphoprep (Ficoll-Paque; Nycomed, Oslo, Norway). Cells were then washed in PBS and slowly resuspended in a 4°C solution of RPMI (Sigma-Aldrich, Gillingham, UK) /10% fetal calf serum

(FCS; Biosera, Ringmer, UK) /10% DMSO (Sigma-Aldrich). Aliquots of 1ml were then transferred to cryotubes (Nuncbrand, Fisher Scientific, Loughborough, UK) and frozen at -80°C for one week and then finally cryopreserved in liquid nitrogen. Normal T and B cells were obtained from buffy coat preparations (British Transfusion Service, Liverpool, UK) which were processed and cryopreserved as for malignant samples.

Cryopreserved CLL, HCL and MCL cells were thawed rapidly at 37°C in a waterbath and then diluted on ice to a volume of 10mls by the slow addition of 9 mls of 4°C RPMI medium supplemented with 0.5% (w/v) BSA (Sigma-Aldrich), 2mM L-glutamine, 100units/ml penicillin and 100μg/ml streptomycin (Invitrogen, Paisley, UK), from now on referred to as culture media. It is essential that this addition of media to 10mls is a gradual process due to the osmotic pressure, which if it were to change too rapidly would cause the CLL cells to lyse. Cells were then pelleted by centrifugation at 4°C for 5 minutes at 500rcf and supernatant discarded. The pellet was then resuspended in 10mls of culture medium and centrifuged as above. These 2 wash steps ensure that the DMSO is removed from the cell suspension as it is cytotoxic at temperatures above 4°C. The pellet was this time resuspended in 1ml of culture medium and the number of cells counted using a haemocytometer (Fischer Scientific) and trypan blue (Sigma) to allow assessment of viability as the trypan blue dye can only be excluded from the live cells which therefore appear clear

and the dead cells appear blue. The cells were then diluted to $2x10^6$/ml, based on the live cell count, and 1ml of this cell suspension added to a well of a 24 well tissue culture plate (Nuncbrand, Fisher Scientific). Wells of these plates were pre-coated with a 95% ethanolic solution of 12mg/ml poly(2-hydroxyethylmethacrylate) (polyHEMA; Sigma-Aldrich) in order to avoid the effects caused by the adhesion of these cells to the bottom of the wells [172]. Cells were then left to 'recover' for 1 hour before time 0 (T0) of culture when stimuli/inhibitors were added.

Normal B cells were cultured in the polyHEMA-coated plates as for CLL, HCL and MCL cells but in RPMI medium supplemented with 10% FCS (Biosera), 2mM L-glutamine, 100units/ml penicillin and 100μg/ml streptomycin . The Jurkat cell line was taken from liquid nitrogen storage, made up to 10ml with the above media and spun as for CLL cells. Cells were resuspended in media and cultured in T25 tissue culture flasks (Becton Dickinson, CA). All cells were cultured in a 37°C incubator (HeraCell, FisherScientific) with 5% CO_2.

2.2.2 Inhibitors and stimuli

All inhibitors were dissolved in DMSO and stored at -20°C. The Lck inhibitor (4-Amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2d] pyrimidin-7-yl-cyclopentane) was used at 1μM, U0126 was used at 10μM, lactacystin was used at 2.5μM, actinomycin D was used at 8nM, cycloheximide was used

at 10μM, flavopiridol was used at 2μM, RO-32-432 was used at 10μM and bisindolylmaleimide I was used at either 1 or 10μM and all were purchased from Calbiochem (Merck Biosciences, Nottingham, UK). Bryostatin-1 was used at 25nM and was purchased from Alexis Biochemicals (Enzo Life Sciences, Exeter, UK). The PKCβII inhibitor (LY379196) used at 1μM was kindly provided by Lilly Research Laboratories (Indianapolis, IN). PMA (phorbol 12-myristate 13-acetate) and bryostatin were purchased from Sigma-Aldrich.

$F(ab')_2$ fragments of goat anti–human IgM (Jackson ImmunoResearch Laboratories; Stratech, Soham , UK) were used at a final concentration of 10μg/ml for crosslinking/stimulating the BCR. Interleukin-2 (Cell Signaling Technology; New England Biolabs, Hitchin, UK) was cultured with cells at 100 international units (IU)/ml.

2.2.3 Western blotting

$2x10^6$ cells were pelleted by centrifugation at 500g for 5 minutes at 4°C, washed 2 times in cold Phosphate buffered saline (PBS) (Fisher Scientific) to remove contaminating BSA and lysed in 100μl clear sodium dodecyl sulphate (SDS) lysis buffer (1% SDS, 10% glycerol, 5mM EDTA, 50mM Tris pH6.8). Lysates were sonicated for 5 seconds using a microson ultrasonic cell disruptor and ultrasonic convertor (Scientific Laboratory Supplies, Nottingham, UK) in order to shear DNA and then heated to 96°C

for 5 minutes using a Grant hotblock (Scientific Laboratory Supplies) to aid denaturation. Determination of the exact concentration of protein present in the lysates was carried out using the DC protein assay (Bio-Rad, Hemel Hempstead, UK) which works in a similar way to the Lowry protein determination method [173]. Lysates were prepared containing 10μg total protein by addition of the relevant quantity of cell sample lysates, clear SDS lysis buffer and finally laemelli buffer [174] to allow addition of β-mercaptoethanol (Sigma-Aldrich) which must be absent from the lysis buffer during protein determination as this reducing agent affects the assay, but present in the final buffer to break disulphide bridges within proteins. These lysates were then loaded onto 10% polyacrylamide gels prepared using the Bio-Rad system (Bio-Rad) and subjected to 30mA electrophoresis for 1 hour using a powerpack (Bio-Rad). 10µl of BenchMark Prestained protein ladder (Invitrogen) was used as a molecular weight marker to allow assessment of the extent of migration of the samples through the gel and to allow identification of proteins according to size. Following completion of electrophoresis, the gel was subjected to transfer to allow protein present in the gel to be transferred onto immobilon membrane (Millipore, Watford, UK) using the Bio-Rad Trans-blot system in transfer buffer (Geneflow, Fradley, UK) for 1 hour at 400mA using a powerpack(Bio-Rad). Immobilon membrane blots were blocked overnight for 1 hour and antibodies prepared in 5% non-fat powdered milk (Tesco)

dissolved in Tris buffered saline (10mM Tris.HCl pH7.4, 0.1M sodium chloride) with 0.1% Tween-20 (TBST).

Following blocking of the blots, they were incubated in the 5% milk/TBST mixture containing the relevant primary antibody. The following primary antibodies were used: anti-Lck mouse monoclonal (Santa Cruz Biotechnology; Insight Biotechnology, Middlesex, UK), anti-ZAP70 mouse monoclonal (Upstate, Milton Keynes, UK) and anti-β actin mouse monoclonal antibody (Sigma-Aldrich). Blots were then washed for 1 hour in TBST to remove unbound antibody and reduce non-specific binding, replacing with fresh TBST every 15 minutes. The blots were then incubated for 1 hour in 5% milk/TBST mixture containing the relevant secondary antibody at a concentration of 80ng/ml (1:5000 dilution). The following secondary antibodies were used: Goat anti-rabbit horseradish peroxidise (HRP) conjugated antibody and goat anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology

Blots were then washed as above for 1 hour in TBST and antibody bound to proteins in the blots visualised using an LAS-1000 chemiluminescence and fluorescent imaging system machine (Fuji, Tokyo, Japan) for measurement of chemiluminescence after treatment of the membrane with enhanced chemiluminescence reagent (Millipore) or ECL-Plus (Amersham Biosciences, Chalfont, UK). The peroxidase-conjugated anti-mouse or

anti-rabbit antibody bound to the primary antibody specific for the protein of interest oxidises luminol in the ECL reagent, emitting light at a peak wavelength of 428nM which is then detected by the machine and displayed as an image which can be analysed and densitometry carried out using the AIDA image analyser software package (Raytek Scientific Ltd, Sheffield, UK).

Stripping of membranes was carried out when a blot had to be reprobed with an antibody produced in the same species. Membranes were incubated at 65°C in stripping buffer containing 62.5mM Tris-HCL (pH 6.8), 100mM β-mercaptoethanol and 2% SDS for 30 minutes in a waterbath. Membranes were washed in TBST for 30 minutes and then blocked in 5%milk/TBST as detailed above. Membranes were then reprobed with the desired antibody as above.

2.2.4 Purification of cells

Negative purification of cells was used in order to avoid stimulating the target cells as may occur during positive purification (i.e. CD19 purification of CLL cells). Purification of CLL, MCL and HCL samples involved incubation of $1x10^7$ cells in 40µl of 4°C degassed purification buffer (PBS pH7.2, 0.5% BSA, 2mM EDTA) for 20 minutes in the presence of 20µl of each of the following FITC-conjugated antibodies CD3, CD14 and CD16 (BD biosciences); these antibodies label T cells,

monocytes, macrophages, neutrophils and NK cells. Cell/antibody suspensions were then centrifuged at 500g for 3 minutes at 4°C, washed in the purification buffer, centrifuged again and resuspended in 80µl purification buffer. 20µl of anti-FITC magnetic beads (Miltenyi Biotech, Bisley, UK) were then added to each sample. Cell/magnetic bead solutions were incubated at 4°C for 15 minutes then pelleted as above, washed in purification buffer, resuspended in 40µl purification buffer and applied to a MiniMACS column (Miltenyi Biotech) in a magnetic bracket (Miltenyi Biotech) for 5 minutes. This causes the CD3, CD16 or CD14 positive cells with the beads attached to remain in the column and allow collection of the unlabelled CD19 positive malignant cells that flow through the column. Three repetitions of 1ml of buffer were added to the column to aid the flow through and collection of the CLL cells in a universal. After this purification procedure CLL, HCL and MCL purity was always greater than 96% B cells as analysed by anti-CD19, CD3, CD16 and CD14 FACS analysis (as detailed in methods section 2.2.6).

Normal T cells were purified from buffy coat preparations using the same method but with anti-CD19, CD16 and CD14 MACS beads instead. Normal B cells were negatively purified using the same column method but utilising two steps. The first step involved incubation of cells with anti-CD3 MACS beads and application to the column to remove a large proportion of the T cells in the sample. This produces a smaller population

of cells in which the B cells make up a larger proportion and so use of a commercially available B cell isolation kit (Miltenyi Biotec) is less costly. The B cell isolation kit contains CD2, CD14, CD16, CD36, CD43, and CD235a (glycophorin A) antibodies conjugated to biotin and anti-biotin beads. After incubation of the antibody mix with the cells, addition of the beads and then application to the magnetic MACS column, as for the CLL purification, a population of >97% normal B cells are collected in the form of column flow-through. Non-B cells, i.e., T cells, NK cells, monocytes, dendritic cells, granulocytes, platelets, and erythroid cells remain in the column.

2.2.5. Quantification of Lck protein

$3x10^7$ CLL cells were thawed as described in section 2.2.1 and immediately resuspended in purification buffer without the 1 hour recovery incubation at 37°C. Cells were purified (section 2.2.4) and purity of the resulting population assessed by FACS analysis. All purified samples were >95% CD19 or CD20 positive indicating that contaminating T, NK, monocytes, macrophages and neutrophil cells had been removed. Cell suspensions were counted and were then divided to produce pellets for RNA extraction of ~$1x10^7$ cells and the remaining cells were used to assess Lck protein quantities. Protein determination was carried out and 10µg total protein from each lysate was subjected to Western blotting. A recombinant GST-tagged Lck protein (Santa cruz Biotechnology) of 0.5, 1

or 4ng concentration was also loaded on the gel to allow calculation of the amount of Lck protein in the lysates as opposed to using densitometry utilising actin as a loading control which would be less reliable.

2.2.5 Polymerase chain reaction (PCR)

All RNA extraction, reverse transcription and PCR protocols are performed using RNase/DNase free equipment, including eppendorphs (Anachem) and filter pipette tips (Anachem). Total RNA was extracted from purified CLL, HCL and normal T and B cells with the RNeasy mini kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. Briefly, $1x10^7$ cells were lysed in 600μl of the provided RLT buffer and then centrifuged at 18000g through a QIAshredder spin column (QIAGEN) to shear the DNA. The flow through was then mixed 1:1 with 70% ethanol and added to an RNeasy spin column. Once centrifuged through the column at 8000g, the RNA (now bound to the column membrane) is first washed by adding 700μl of the provided RW1 wash buffer. The spin column is centrifuged at 8000g and the flow-through is discarded. Next, 500μl of the provided RPE buffer is added to the column and once again the column is centrifuged at 8000g. The RPE wash step is then repeated but the centrifugation step is increased to 2 minutes to dry the spin column of any remaining ethanol. The 2ml collection tube is then replaced with a 1.5ml collection tube and 50μl of RNase free water is used to elute the pure RNA from the spin column by centrifuging at $\geq$8000g for 1 minute. RNA

concentration and purity was measured at 260/280nm using a NanoDrop machine (Thermoscientific from Labtech International Ltd., Ringmer, UK).

1μg of purified RNA was reverse transcribed to cDNA in a 20μl reaction containing 1µl (200U) Molony murine leukemia virus (M-MLV) reverse transcriptase (Promega, Southampton, UK), 1µl (500ng) oligo$(dT)_{15}$ primer (Promega), 1μl of 10mM dNTPs (Promega), 0.5µl (25U) Recombinant RNasin® Ribonuclease Inhibitor (Promega) and 4µl M-MLV 5x reaction buffer (Promega). Initially, the RNA was added to the oligo$(dT)_{15}$ primer, made up to 13.5μl with H_2O, and heated to 70°C for 5 minutes. Samples were then cooled on ice and the remaining reagents were added. The reaction is then incubated at 37°C for 1 hour followed by heating at 65°C for 10 minutes to deactivate the reverse transcriptase enzyme.

The Lck specific PCR primers were; transcript I forward (PI): 5'-GGATCCCAGGATCTCACAATC-3', transcript II forward (PII): 5'-GGACCATGTGAATGGGGCCAGAGG-3', reverse primer for both transcripts (PIII): 5'-GCACCTCAGAGCCATTTCG-3' (Eurofins MWG Operon, London, UK) and were taken from [115]. Primers were received in lyophilized form, and were reconstituted to 100pmol/µl in H_2O and then diluted to provide a 20pmol/µl working stock.

All semi-quantitative PCR reactions were performed on ThermoHybaid PxE 0.5 machines (Thermoscientific) and contained: 16.75µl H_2O, 5µl 5x Green GoTaq Flexi buffer (Promega), 1.5µl 25mM $MgCl_2$ (Promega), 0.5µl of each of the forward and reverse primers (final concentration 0.4pmol/µl), 0.5µl 10mM dNTPs (200µM final concentration), 0.5µl of the cDNA template and 0.25µl GoTaq DNA polymerase (Promega). The optimised PCR conditions were 10 minutes at 95°C followed by 30 cycles of 94°C denaturation, 67°C annealing and 72°C extension for 30 seconds each. PCR products were subjected to agarose gel electrophoresis (1% gel at 140 constant volts for 1 hour) in the presence of ethidium bromide and visualised using a Fujifilm FLA-5000 (Fuji). A 1% agarose gel contains 1.5 grams of agarose (Web Scientific Ltd, Crewe, UK) boiled until dissolved in 100mls of H_2O in a conical flask using a microwave. 15mls of 10x Tris-acetate-EDTA (TAE; Sigma) buffer was then added followed by 35mls H_2O and 1.5µl of a 10mg/ml ethidium bromide solution (Sigma). The suspension was mixed, poured into a horizontal gel plate and two 20 well combs were placed into the gel and left to set for 20mins. The combs were then removed, the gel placed in a running tank (Scie-Plas, Fisher Scientific) and 1x TAE buffer containing 100µg/ml ethidium bromide was added. 5µl of the PCR reaction was loaded into each lane and the first and last lane of every gel contained 10µl of 100bp ladder (New England Biolabs). Gels were visualised using a Fluorescent Image Analyser FLA-5000 (Fujifilm from Raytek Scientific Ltd., Sheffield, UK).

2.2.6 Quantitative Real-time PCR

Primers were designed using sequence information on the NCBI Entrez nucleotide database (records NM_001042771.1 and NM_005356.3, Homo sapiens lymphocyte-specific protein tyrosine kinase transcript variant 1 and 2 respectively as detailed in the appendix). Primers were designed to amplify the coding region and would therefore amplify Lck transcripts initiated from both the proximal and distal promoter, therefore giving a reflection of the amount of total Lck mRNA present. The forward primer sequence was 5'-GAGGCTGTGCTGGAAGGAG-3' and the reverse primer sequence was 5'-TGTGGTCTCAGGAAATGGGAG-3' (Eurofins MWG Operon); the product has a length of 383 nucleotides. qRTPCR reactions were performed in Mx3000P 96-well qPCR plates (Agilent Technologies, South Queensferry, UK) with 8x strip optical caps (Agilent Technologies) on a Stratagene Mx3005P qPCR machine (Agilent Technologies). Each PCR reaction comprised of 0.25µl cDNA template, 0.5µl (10pmol) of each forward and reverse primer, 11.25µl H_2O and 12.5µl 2x DyNAmo SYBR Green qPCR master mix (New England Biolabs). Optimised PCR reaction conditions were 10 minutes at 95°C followed by 45 cycles of 95°C denaturation, 67°C annealing and 72°C extension for 30 seconds each. The products were measured using a 7 second 81°C read step at end of each cycle. A final dissociation read section (multiple product reads from 55-95°C) at the end of the PCR verified the correct product amplification read temperature. To further

confirm the specificity of the primers and the presence of only 1 PCR product of 384 bp in length, 5µl of the PCR reaction was mixed with 1µl 6x Orange G loading buffer (1.5grams Ficoll 400 (Sigma), Orange G Dye (Sigma) dissolved in 10mls H_2O) and was ran on a 1.5% agarose gel.

2.2.7 Fluorescent activated cell sorter (FACS) analysis

The purity of the CLL cell populations was determined by flow cytometry using a Becton Dickinson FACSCalibur machine and FITC-labelled anti CD3, CD19 or CD20 antibodies and IgG controls (BD Biosciences). Lck was detected using an unlabelled rabbit primary antibody (Santa Cruz Biotechnology) and a phycoerythrin (PE) labelled secondary antibody (Invitrogen). When analysing surface expressed proteins, $1x10^6$ cells were incubated for 20 minutes on ice, in the dark, with 10µg/ml final concentration of antibody. When analysing internal proteins, such as Lck, $1x10^6$ cells were fixed using 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes, then permeabilised using 0.1% TX100 (Sigma-Aldrich) for 5 minutes, and the free paraformaldehyde was quenched using 50mM glycine (Sigma-Aldrich) for 5 minutes. The cells were then resuspended in the antibody at 10µg/ml final concentration in 100µl PBS and incubated as above. The cells were centrifuged at 500g for 5 minutes at 4°C and washed using 100µl PBS between each incubation step. Cells were finally resuspended in 100µl PBS to which 100µl FACS flow (BD biosciences) was added. This sample was then analysed by FACS analysis.

2.2.8 Cell survival analysis

1ml of $2x10^6$/ml cells in culture media were incubated with various stimuli in the presence or absence of 1μM of the Lck inhibitor for 48 hours and the relative proportion of alive or dead cells assessed using a 3,3-dihexyloxacarbocyanine iodide/propidium iodide ($DiOC_6$/PI) apoptosis assay. $4x10^5$ cells were harvested from culture in 200μl culture medium and added to 200μl of 80nM $DiOC_6$/PBS (Sigma-Aldrich). This was then incubated at 37°C for 20 minutes. 400μl of 10μg/ml PBS/PI (Sigma-Aldrich) solution was added and incubated on ice, in the dark, for 30 minutes. $DiOC_6$ is a marker of membrane integrity and alive cells are $DiOC_6$ positive and fluoresce on the FL1 wavelength of the FACS machine. Early apoptotic cells do not contain as much DiOC6 and are negative of FL1 fluorescence. Apoptotic cells are also FL1 negative but have bound PI to the exposed DNA and are therefore FL2 positive.

2.2.9 siRNA

1 x 10^6 CLL cells were resuspended at a concentration of 2 x106 per ml and treated with 100nM of either Lck-specific siRNA (Ambion; Applied Biosystems, Warrington, U.K), a mixture of 4 SMARTselection-designed siRNAs targeting Lck (Dharmacon RNA Technologies; Thermo Fisher Scientific), or nonspecific control siRNA (Silencer negative control #1 siRNA, Ambion). siRNA was diluted in 100μl culture medium and pre-incubated with 6μl HiPerFect Transfection Reagent (QIAGEN) for 10

minutes at room temperature to allow formation of transfection complexes. These siRNA-HiPerFect complexes were then added dropwise into the cell suspension with gentle swirling of the plate to allow uniform distribution of the transfection complexes. Cells were then cultured for 48 hours before harvesting and analysis of protein content by SDS-PAGE and Western blot. In addition, in the case of the experiments where 2 hits of siRNA were used, the cells were washed after 48 hours, resuspended in fresh culture media and subjected again to the siRNA treatment. Cells were cultured for a further 48 hours and then analysed as above. A control FITC-labelled siRNA (Ambion) was used to assess the transfection rate of the CLL cells and this was analysed using FACS analysis and showed an average uptake of 88%.

2.2.10. Statistical analysis

SPSS or Excel were used to analyse the statistical significance of data. The Mann-Whitney U or Students T test was used and $p<0.05$ was considered statistically significant. All averages are expressed as the mean +/- the standard deviation.

2.3 Results

2.3.1 Expression of Lck mRNA in CLL cells

The results of the RT-PCR analysis into the presence of Lck mRNA in CLL, Jurkat, HCL and normal B cells are shown in figure 2.1a and 2.1b.

Figure 2.1a is obtained by using transcript 1 primers that amplify transcripts initiated from the proximal, type I promoter. This figure shows the amplification of 3 bands; transcription product Ib (165bp) which is the expected product that is transcribed from the type I promoter (Figure 2.1.d), and products Ia (263bp) and preIa (567bp) which are likely to be splice variants of the transcript from the type I promoter. These products have been noted in a previous publication investigating Lck expression [115], and could be expected to be present based on the structure of the *lck* gene. Thus, the Ia and Ib products are clearly present in the Jurkat T cell line, whereas they are absent in normal peripheral B cells and in HCL cells. In CLL cells the Ia and Ib products were variably present depending on the case used. All of the cases used in this study expressed the preIa product.

Figure 2.1b is obtained by RT-PCR using transcript II primers that amplify transcripts initiated from the distal promoter [115]. All cases of CLL, as

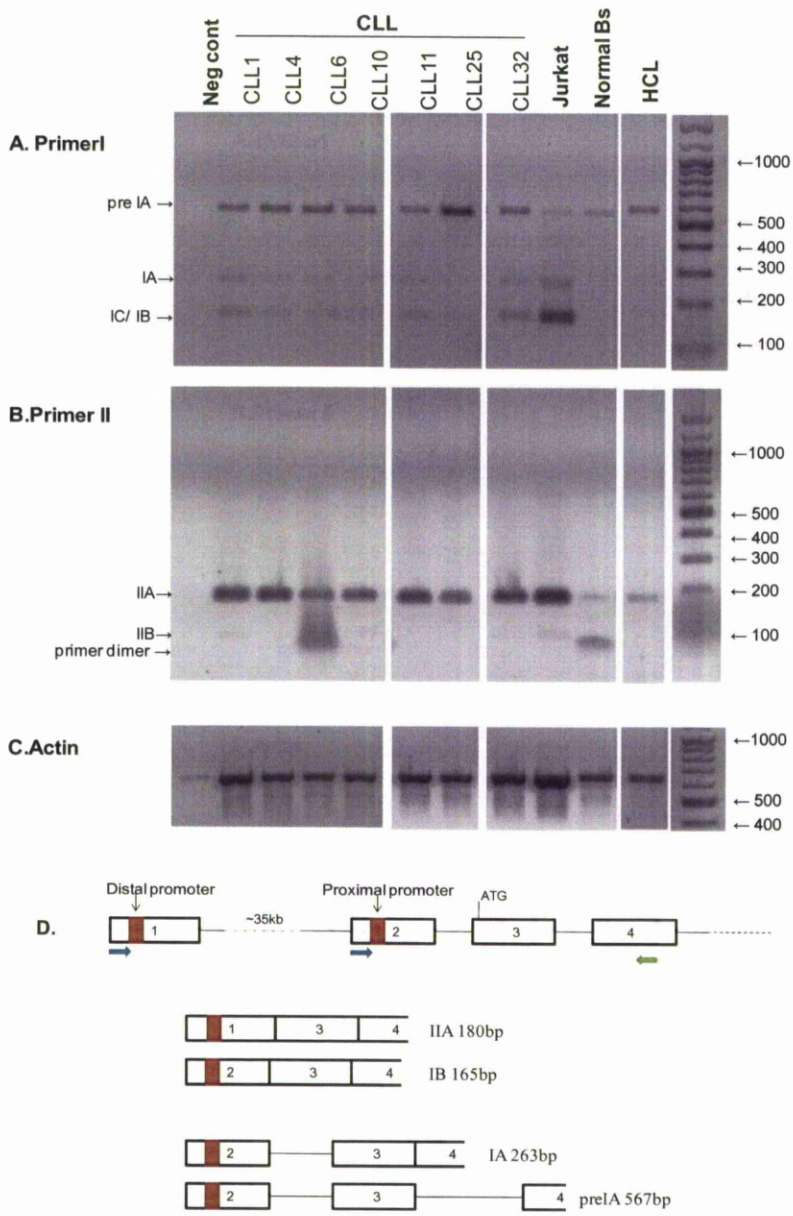


Figure 2.1 The presence of the different transcripts in CLL cells, Jurkat T cell line, normal peripheral blood B cells and HCL cells. CLL, HCL and normal peripheral B cells were highly purified. cDNA was subjected to PCR using either transcript I (part a) or II (part b) specific primers (as detailed in section 2.2.5) or actin primers (part c). PCR products were run on a 1.5% agarose gel. The size of different products are shown in relation to a 100bp DNA ladder. Part d is a schematic of the gene structure and PCR products for transcription from both type I and type II promoters. Results are representative of two experiments.

did Jurkat T cells, contained the expected 180bp PCR product (IIA). This product is only weakly present in normal peripheral B cells and HCL cells. The band designated IIB is present in cases CLL1 and CLL6 and the Jurkat cell line. The identity of this band has been confirmed by sequencing, and is derived from the Lck transcript [115]. The more pronounced band observed in case CLL6 and in normal B cells migrates to a similar position on the gel (fig 2.1b) and could be a primer-dimer.

Taken together, the above results demonstrate the presence of Lck mRNA in CLL cells. These results further suggest that transcription of the Lck gene to generate mature mRNA takes place from the distal promoter, and, in some cases of CLL, also from the proximal promoter.

2.3.2 Quantification of total Lck mRNA

In order to assess if the amount of Lck protein is regulated at the transcriptional or post-transcriptional level and if the levels of Lck mRNA correlate with the amount of Lck protein, quantitative real time PCR (qRTPCR) was used. Primers were designed to amplify product from the coding region of the gene and would therefore amplify transcripts initiated from both the distal and proximal promoters.

Purified CLL cells from cases that contained different levels of Lck protein (as assessed by Western blotting in figure 2.3) were selected. Normal T

cells and B cells purified from 3 different buffy coat samples were also analysed to allow comparison. One HCL case was used as an example of a cell type that contains very low levels of Lck protein (figure 2.3).

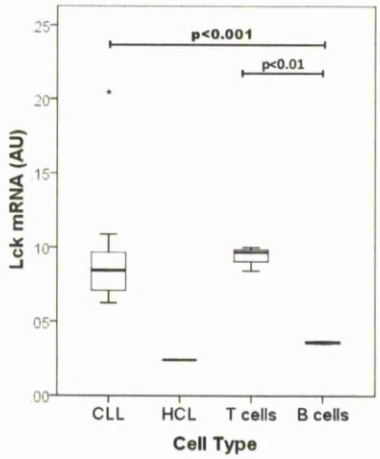
Figure 2.2 shows the qRTPCR results of the different cell types and includes analysis of whether levels of mRNA correlate with levels of protein. CLL cells contained significantly more Lck mRNA than normal B cells ($p<0.001$). The levels of Lck mRNA in CLL cells were very similar to normal T cells ($p=0.921$). HCs contained the lowest levels of Lck mRNA, even lower than that expressed in the normal B cells. This low Lck mRNA expression of HCL is to be expected based on the result in figure 2.3 that shows that these cells contain very low levels of Lck protein.

We next examined Lck protein expression in different CLL cell clones.

2.3.3. Expression of Lck protein in CLL cells

To examine Lck protein expression in CLL we first compared Western blots of Normal T and B cells, the malignant cells of hairy-cell leukaemia (HCL) and mantle-cell lymphoma (MCL) cells with those of purified CLL cells. Figure 2.3 shows that T cells and CLL cells express easily detectable amounts of Lck protein, whereas HCL cells do not. B cells from normal peripheral blood contain very low levels of Lck protein that may represent

A.



B.

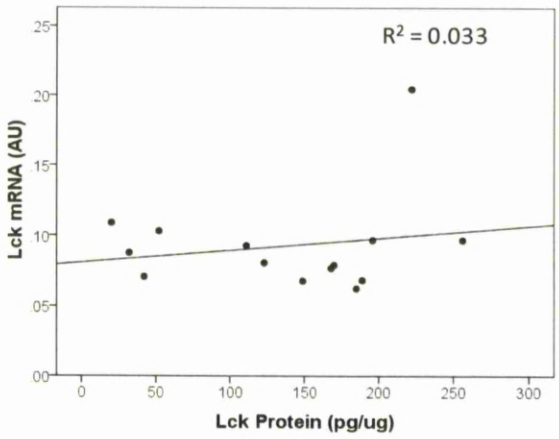


Figure 2.2. Levels of Lck mRNA in CLL and normal T cells are similar and are higher than the levels in normal B cells and HCL cells. **A**; Amount of Lck mRNA normalised to GAPDH in 14 CLL cases, 1 HCL, 3 normal peripheral blood T cell samples and 2 normal peripheral blood B cell samples. One CLL is an outlier represented on the boxplot as a single dot which contained 20.5 arbitrary units of Lck mRNA. Statistical significance was determined by the Mann-whitney U test. **B**; correlation of Lck mRNA levels with protein expression in 14 CLL samples. Statistical significance was determined by assessment of linear regression using Excel.

the B-1 cell population. MCL cells variably expressed Lck depending on the case. These results were reproducible using different antibodies to Lck (data not shown). Thus, in agreement with Majolini et al [4], our results demonstrate that CLL cells express Lck protein.

We next examined Lck expression in the lysates of 45 different CLL clones where the malignant cells had been highly purified. Figure 2.3 shows that Lck expression in a selection of these cases varied dramatically. To quantitate the levels of Lck protein in CLL cells we used a Western blot-based technique whereby Lck levels in CLL cell lysates were estimated against standard amounts of recombinant Lck protein. Figure 2.3 shows that there was a linear relationship between 0.5 and 4ng of total loaded recombinant Lck. Lck expression in CLL cell lysates was estimated from 10μg of total cellular protein and is reported as pg Lck/μg total cellular protein. This method was reproducible using mouse anti-Lck (Santa Cruz clone 3A5) and rabbit anti-Lck (Cell Signaling clone 73A5) antibodies (Figure 2.3). Table 2.1 shows the amount of Lck protein in the malignant cells from each CLL clone tested. We found that there was a range of Lck expression from 20 to 256 pg/μg total cellular protein. Interestingly, a comparison between Lck protein and mRNA levels in CLL cells shows that despite the very large variation in protein, there is relatively small variation in mRNA. Moreover, the levels of Lck mRNA do not correlate with the levels of protein (Figure 2.2B). These data suggest

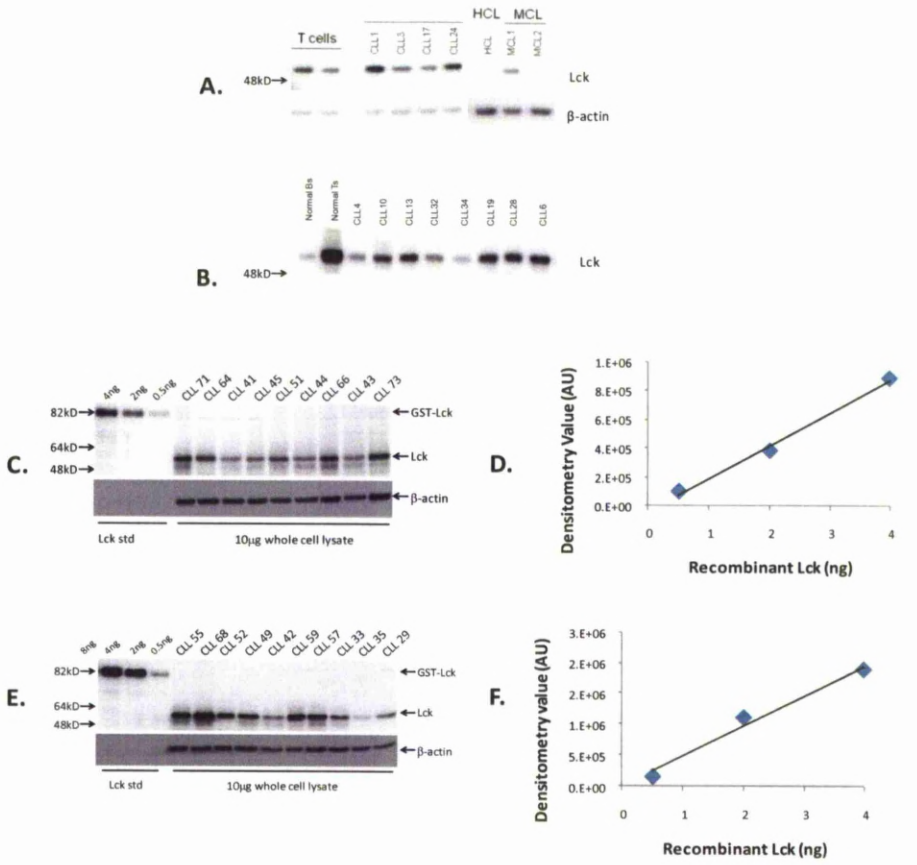


Figure 2.3. Expression of Lck protein in CLL, HCL, Mantle cell lymphoma (MCL) and normal peripheral blood T and B cells. All cell types were purified by removal of contaminating cells using negative purification (methods section 2.2.4) prior to lysis, protein determination and Western blotting. Actin was used as a measure of sample loading and the actual amount of Lck in different samples in **C** and **E** were calculated by comparison with 0.5, 2 or 4ng of recombinant Lck protein standard (Lck std). The analysis of the standards are shown in **D** and **F**. Results are representative of two experiments.

CLL case	Lck (pg/ µg)	c-Abl (pg/mg)	ZAP70 (relative)	Lyn (relative)	WBC	IgVH mut	gene usage	class	stage
CLL 35	20	2	30.21		142	0.4	3-48/	M	
CLL 30	32	17	9.84		240	0.0	5-51/	A	
CLL 36	40	68	27.91		118.5	3.6	3-73/	M	
CLL 37	42	19	4.30		242	12.3	3-53/	G	
CLL 38	49	87	17.76		126.5	0.0	3-09/	M	C
CLL 39	52		43.68		184	0.0	3-30/	M	
CLL 40	55	44	29.74		183	0.3	2-70/	M	
CLL 41	58			7.7	23.3	1.35	3-48/	M	A
CLL 22	58	58	23.00		155	5.4	4-34/	G	
CLL 29	61		5.63		158	8.9	4-34/	M	C
CLL 42	65			15.4	412	0	DP8	M	
CLL 43	75			8.4	86.8	1.09	3-66/	M	B
CLL 44	75			10.1	49.3	-	-	-	A
CLL 33	81			17.2	382	0	DP8	M	
CLL 45	101			7.5	215	4.47	4-43/	M	A0
CLL 46	107	25	39.34		128	3.2	3-48/	M	
CLL 47	109	23	32.37		116	6.1	3-23/	G	A
CLL 48	110				24.7	8.5	3-13/	M	A0
CLL 49	111			16.3	66.4	11.11	3-48/	M	C
CLL 17	117		43.36		427	0.0	1-18/	M	B
CLL 50	123	61	29.50		124.7	0.0	1-69/	M	
CLL 51	123		42.54	11.5	147	0	4-34/	M	A
CLL 52	127			16.1	124	6.12	V3-49	G	B
CLL 53	138	101	9.33		110.7	8.9	4-34/	G	
CLL 54	142	31	29.10		187	0.0	4-31/	M	B
CLL 55	145			13.9	182	6.45	1-69/	M	B
CLL 56	149	57	41.06		266	0.3	3-49/	M	
CLL 57	168			20.9	-	0.35	1.46	M	
CLL 58	170	10	10.23		210	8.6	3-09/	M	B
CLL 59	181	59	9.43	18	165	4.6	4-34/	M	
CLL 60	182	49	42.20		127	0.0	1-69/	M	
CLL 61	185				165	4.56	4-34/	M	A
CLL 62	185	103	42.08		71.5	-	-	-	
CLL 63	187	32	8.19		165	4.6	4-34/	M	
CLL 64	187			10	54	6.12	3-72/	M	
CLL 65	189	15	3.29		196	3.1	3-30/	M	
CLL 66	196			11.7	127	-	-	-	B
CLL 67	199	85	4.24		150	5.2	3-48/	M	
CLL 68	211			9.9					
CLL 69	213		11.32		98.8	4.4	3-07/	M	A
CLL 70	219	82	6.73		175	6.6	1-69/	M	
CLL 71	222			12.9	174	1.75	4-59/	M	A
CLL 72	236	82	14.80		105	1.8	4-59/	M	
CLL73	256	31	26.97	12	76.6	1.7	4-61/	M	BII

Table 2.1 Levels of Lck, ZAP70 or Lyn protein and characteristics of the cases used. Levels of Lck are expressed as pg/µg total cellular lysate in comparison to a recombinant standard. Lyn and ZAP70 levels are expressed as arbitrary units in comparison to β-actin. WBC is the whole blood cell count of the sample (expressed as 10^9cells/litre).

that post-translational regulation of Lck expression may be important in CLL cells.

A previous study has shown that the SFK Lyn is overexpressed in CLL cells [132]. Figure 2.4 shows that in contrast to the considerable variability of Lck expression between CLL cell clones, Lyn expression was relatively consistent. This suggests that the mechanisms that control Lck and Lyn protein expression in CLL cells are different.

ZAP70 is a major substrate of Lck in T cells. CLL cells also express ZAP70, but the expression of this protein varies between malignant cell clones [63]. In particular, high expression of ZAP70 is associated with low levels of IgVH mutation. To investigate the possibility that Lck and ZAP70 expression in CLL cells may be similarly controlled, we next compared the expression of these two proteins in the malignant cells of the CLL clones we analysed. Figure 2.4 shows, in agreement with the work of others [65], that ZAP70 expression varied between CLL cases. Moreover, our data also agree with the published results of others; ZAP70 expression was higher in CLL cases with unmutated IgVH (Figure 2.4.B.). However, when we correlated ZAP70 expression with that of Lck we found there was no significant relationship (figure 2.4.C.).

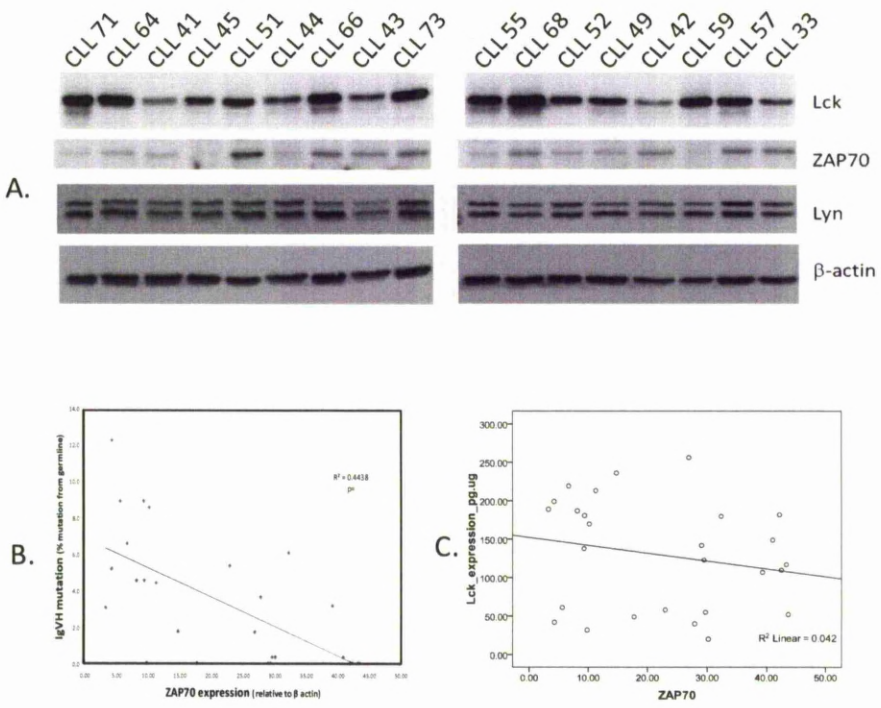


Figure.2.4. Comparison of Lck protein levels with ZAP70 and Lyn. 17 representative CLL cases negatively purified were subjected to Western blotting for Lck, ZAP70, Lyn or *β*-actin as a loading control (**A**). Densitometry was performed and the amount of each protein was normalised to actin and comparison of the levels with each other and with markers of disease such as IgVH mutation were made. **B**; IgVH versus ZAP70 levels and **C**; Lck protein versus ZAP70.

ZAP70 expression is a useful marker for CLL disease prognosis because CLL-cell clones bearing unmutated IgVH genes generally express high levels of this protein. When we compared Lck expression in CLL-clones bearing unmutated IgVH genes with those bearing mutated IgVH genes we found no significant difference (Figure 2.5A). This result was expected based on the previous finding that there was no significant correlation between ZAP70 and Lck.

We next examined Lck expression in CLL clones bearing different VH gene segments. Figure 2.5B shows that Lck expression could not be correlated with particular gene usage. Furthermore, a comparison of Lck expression in CLL cells that had undergone Ig class switch showed no difference in protein expression between IgM and IgG bearing cases (figure 2.5C). Finally, we compared Lck expression with white blood cell count. This analysis showed no correlation between Lck expression and tumour burden in CLL (Figure 2.5D).

Taken together, the above results show a wide variation in Lck expression between CLL cell clones that has no relation to prognostic indicators of disease. This suggests that Lck is likely not to have value in CLL disease prognosis. Furthermore, the above data provide no hint to the processes that drive Lck expression in CLL cells.

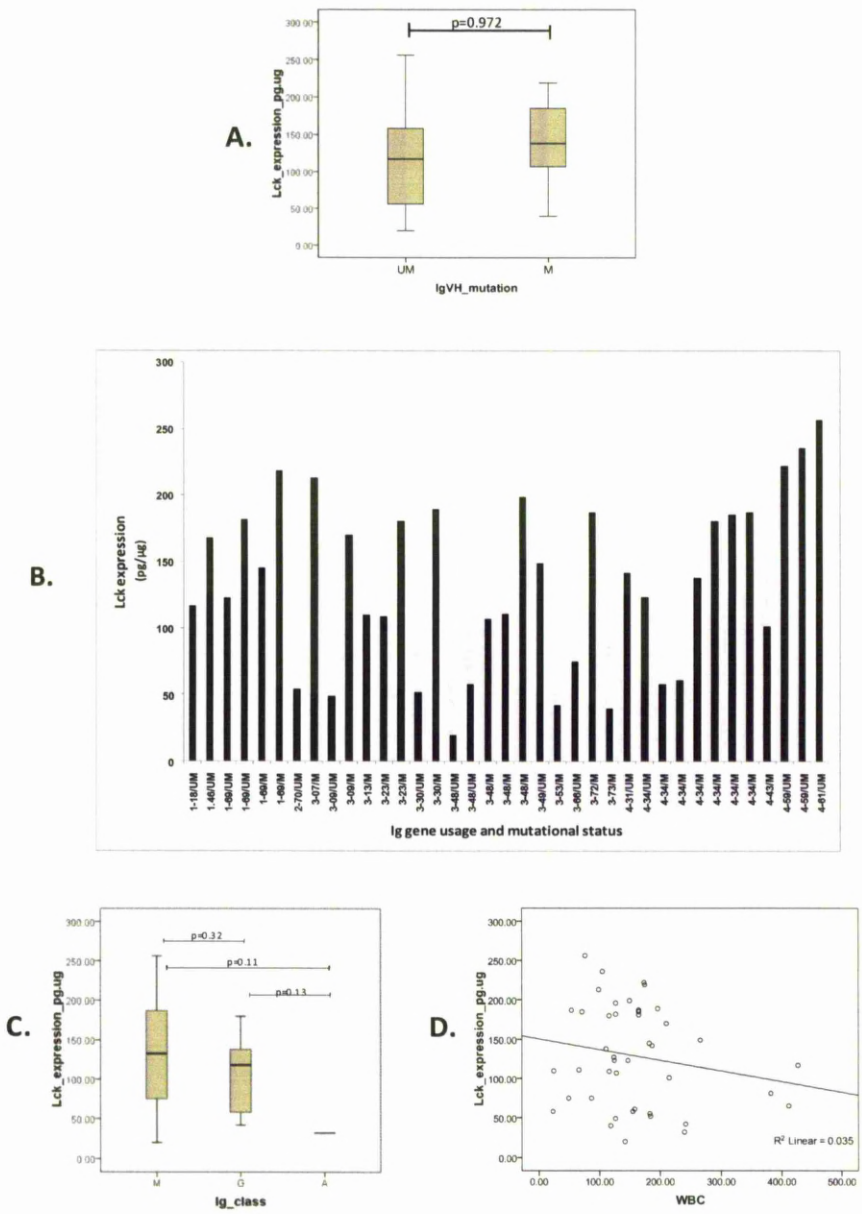


Figure 2.5. Comparison of Lck protein levels with markers of CLL disease prognosis. A; IgVH mutation defined as unmutated (UM) if the % deviation from the germline sequence is <2. **B**, immunoglobulin gene usage and mutational status. **C**, Immunoglobulin class (note only 1 IgA case was analysed). **D**; whole blood cell count (WBC).

2.3.3.1 Detection of Lck in CLL cells by flow cytometry

To further explore Lck protein expression in CLL cells, and to confirm the results generated by Western blot, I used flow cytometry. This method has the advantage of being able to examine the appropriate cell population in unpurified cell preparations. The major disadvantage of this technique is that protein expression is semi-quantitative and small differences are not easily distinguishable.

Figure 2.6 shows that Lck can be detected by FACS analysis in both T and B cells in CLL cell samples. Furthermore, this figure also shows that the majority of Lck positive cells in the CLL cases are B cells. These data validate the Western blot analysis of Lck expression in CLL cells.

During our experiments examining Lck protein expression in CLL cells, I often observed the appearance of a slower migrating minor band in some but not all of the CLL cases I analysed (Figure 2.7, CLL cases 10 and 16 versus 2 and 21). Several reports have indicated that the appearance of such a band is associated with serine/threonine phosphorylation of Lck. This post-translational modification of Lck seemed to be important and was therefore further investigated in the second part of this Chapter.

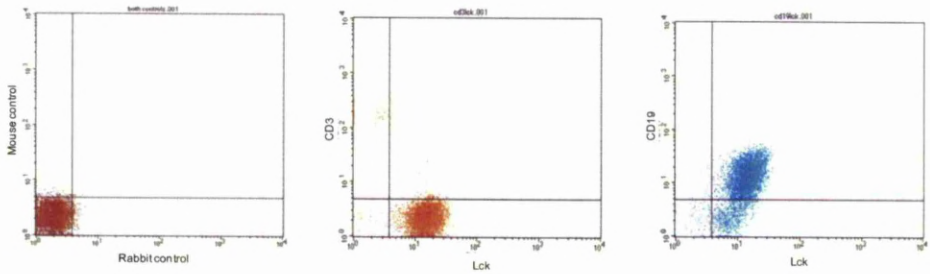


Figure 2.6. Expression of Lck protein in CD19 and CD3 positive cells in non-purified CLL samples. FACS analysis of cells stained with anti-Lck antibody and either CD19 or CD3 was performed on whole, non-purified, CLL samples to compare the amount of Lck expressed in CD19 positive B cells and CD3 positive T cells (methods section 2.2.7). Representative of 5 CLL cases.

2.3.4 Investigation into the mechanism and function of Lck serine phosphorylation in CLL cells.

2.3.4.1 Appearance of the slower migrating Lck band is associated with the stimulation of CLL cells with PMA and signalling through the BCR

It is well established that activated Lck can be phosphorylated on serine 42 and 59 by enzymes such as PKC and ERK which results in the generation of an Lck band of apparently larger molecular size (60kDa). This band has been designated p60 [100]. In T cells the appearance of the p60 band is often taken as an indication of Lck activation because of its association with engagement of the TCR [175]. Appearance of the p60 band can also be stimulated using PMA, but whether this indicates full activation of Lck is not clear.

Figure 2.7 shows Western blots of Lck in four CLL samples, two in which the 60kDa band was constitutively present (CLL10 and CLL16) presumably because of *in vivo* cell stimulation, and two that did not contain this band (CLL2 and CLL21). When the cells were stimulated by PMA this caused a rapid shift of a large proportion of the p56 band to the 60kDa position regardless of whether or not the cells already contained this slower moving band. Incubation for 1 hour with either the specific PKCβ inhibitor (LY379196) or a general inhibitor of PKCs (Ro32-0432) caused

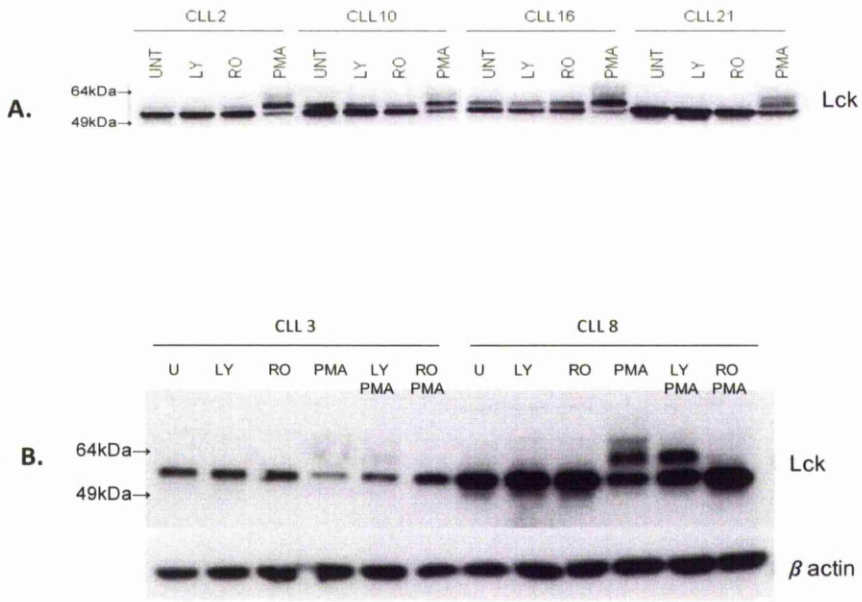


Figure 2.7. Lck migration shift in response to PMA is PKC dependent

A. 4 CLL samples were cultured with a PKC beta inhibitor (LY379196) at 1μM, a PKC general inhibitor (Ro-32-0432) at 10μM for 55 minutes with or without PMA at 100nM for 5 a further minutes to stimulate PKCs. Lysates were subjected to Western blotting for Lck (as detailed in methods section 2.2.3). **B.** 2 CLL cases were preincubated with the same inhibitors (as for A) for 55 minutes and then stimulated with 100nM PMA for 5 minutes and then Western blotted for Lck and actin. Results are representative of two repeat experiments.

no clear reduction of the constitutively present 60kDa band (figure 2.7A). These results suggest that the mechanisms regulating the shift in Lck mobility in resting CLL cells might be different from those that are stimulated by PMA to result in the slower migrating band.

To investigate the nature of the PMA-induced shift in Lck mobility I tested the effect of the above PKC inhibitors on the induction of this shift (figure 2.7B). In these experiments a lower percentage polyacrylamide gel was used to analyse the Lck shift than was used in figure 2.7A. Reducing the acrylamide concentration allowed for better separation of the Lck bands and resulted in the appearance of a third band migrating above p60. The appearance of this band is reported to indicate phosphorylation of Lck at both serine 42 and 59 [97]. Pretreatment of CLL cells with LY379196 inhibited the appearance of this higher molecular weight band, whereas pretreatment with Ro32-0432 inhibited the appearance of both bands. These results suggest that the PMA-induced shift in Lck mobility is partially mediated by activation of PKCβ, but that the majority is likely to be mediated by other, possibly novel, isoforms of PKC.

The above data suggest that Lck may associate with PKCs, where these latter enzymes are responsible for serine phosphorylation of activated Lck. However, in some studies Lck has been found to be associated with and phosphorylated by ERK [175]. It is therefore possible that Lck activation

may also involve ERK and that either PKC or ERK, or both, are involved in post-translational modification of Lck. Since both ERK and PKC play a role in BCR signalling, the appearance of the p60 band in response to BCR cross-linking on CLL cells in the presence of PKC and MEK inhibitors was studied next.

Figure 2.8B shows that BCR stimulation of CLL cells induces appearance of the p60 Lck band. Pretreatment of the cells with inhibitors of PKC (Ro32-0432 or bisindolylmaleimideI, both used at 10μM), or the MAP kinase pathway (U0126), or of Lck itself inhibited the formation of this band. These results confirm a role for PKC and ERK in the BCR-induced change in Lck mobility, and further suggest that BCR mediated activation of Lck may also be involved. As demonstrated for PMA-induced p60 formation, PKCβ and possibly also PKCε may play small roles because treatment of CLL cells with 1μM bisindolylmaleimideI, which inhibits these PKC isoforms at this concentration, partially inhibited the disappearance of the p56 band.

To investigate the role of ERK we analysed the level of phosphorylation of this protein in response to BCR crosslinking as a function of the appearance of the p60 band of Lck. Figure 2.8A shows that BCR engagement induced an increase in the level of pERK in all the cases

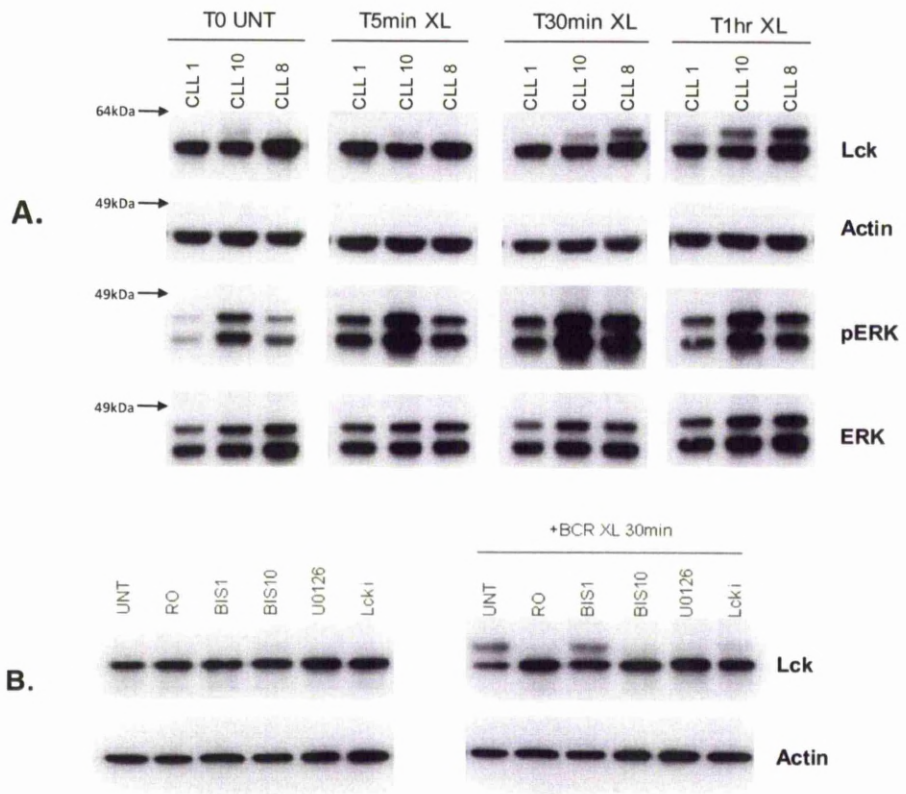


Figure 2.8. Unstimulated p60Lck is present in CLL cases with high baseline pERK levels and increases in response to BCR crosslinking in a PKC, MEK and Lck-dependent manner. A. Levels of Lck, phosphorylated ERK (pERK), total ERK and β-actin in four CLL cases subjected to 5, 30 or 60 mins of 10μg/ml anti-IgM stimulation(XL). Representative of two experiments. **B.** One CLL clone (CLL8) was pre-incubated with the indicated inhibitor for 1 hour, then stimulated with $F(ab')_2$ anti-IgM at 10μg/ml final concentration for 30 minutes (+ BCR XL). The general PKC inhibitor Ro-32-0432 was used at a 10μM concentration (RO) and the PKCϵ-specific inhibitor bisindolylmaleimideI at concentration of 1 or 10μM (BIS 1/10). The MEK inhibitor U0126 was used at 10μM in order to inhibit ERK activation and the Lck inhibitor used at 1μM. Representative of five cases.

tested. This increase was apparent at 5 minutes following addition of the crosslinking antibody, and was maintained over the 1 hour duration of the experiment. Appearance of the p60 Lck band only became apparent at 30 minutes following BCR stimulation. These results demonstrate a time delay between the kinetics of ERK phosphorylation and the appearance of the p60 Lck band. The reason for this time delay is unclear, but it suggests that active PKC and ERK must be brought into the proximity of Lck in order for the p60 Lck band to be formed and that this is a secondary effect of BCR crosslinking.

Interestingly, the presence of the p60 Lck band in resting CLL cells seemed to be associated with high levels of pERK (Figure 2.8A). Based on the above results, and on the known role of the BCR in CLL pathobiology [53], the appearance of the p60 band may be the result of prior stimulation of the CLL cells *in vivo*.

2.3.4.2 Formation of the p60 Lck band in CLL cells is associated with serine phosphorylation

Previous reports have indicated that Lck can be phosphorylated on serine 42 and 59 and that this results in the generation of the p60 Lck band [97, 100]. I next investigated whether the stimulated appearance of this band in CLL cells was also associated with serine phosphorylation.

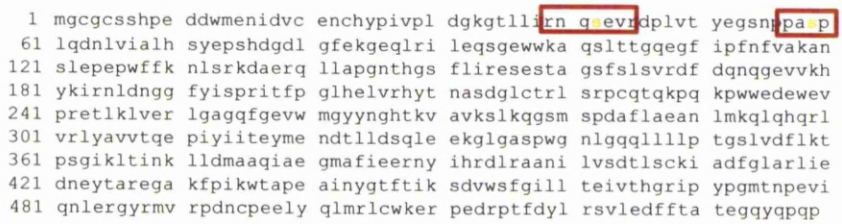


Figure 2.9. Amino acid sequence of Lck highlighting the serine 42 and 59 PKC and MAPK phosphorylation consensus sequences.

Figure 2.9 shows a schematic of the amino acid sequence of Lck that includes the phosphorylation sites at serine 42 and 59 (highlighted in yellow). Analysis of the peptide sequence surrounding serine 42 shows that this is a consensus phosphorylation sequence for PKC, while the peptide sequence surrounding serine 59 corresponds to the consensus phosphorylation sequence for ERK (red boxes) [97]. Since formation of the p60 Lck band in response to PMA or BCR crosslinking can be inhibited by U0126 and Ro32-0432 these data indicate that phosphorylation at serine 42 and 59 of Lck are likely to occur in CLL cells.

To investigate actual serine phosphorylation of Lck I used an anti-phospho serine antibody that specifically recognises serine that is phosphorylated in the context of a PKC consensus substrate sequence. I first investigated the effects of PKC activation on Lck phosphorylation. Figure 2.10 shows that stimulation of CLL cells with the PKC agonist bryostatin resulted in the appearance of the p60 Lck band in whole cell lysates. Figure 2.10 also

shows that the anti-PKC substrate antibody immunoprecipitated protein bands that were reactive with the Lck antibody. Pretreatment of CLL cells with 1μM bisindolylmaleimideI resulted in the partial inhibition of bryostatin-stimulated p60 formation in whole cell lysates, and complete inhibition of the top band within the anti-PKC substrate immunoprecipitates. Taken together, these results indicate that bryostatin stimulates the formation of the p60 Lck band in CLL cells through PKC-mediated serine phosphorylation of Lck.

I performed the same experiment with U0126 to investigate the role of ERK. Figure 2.10B shows that the treatment of CLL cells with U0126 resulted in a reduction in the molecular weight of the Lck reactive band that was immunoprecipitated with the anti-PKC substrate antibody from bryostatin stimulated CLL cells. This indicates that active ERK is likely to contribute to the phosphorylation of Lck in CLL cells. Figure 2.10B also shows that treatment of CLL cells with 10μM bisindolylmaleimideI results in the complete inhibition of anti-PKC substrate antibody immunoprecipitation of Lck reactive bands from bryostatin treated CLL cells. This corresponds to the complete inhibition of p60 Lck formation in whole cell lysates.

I also analysed Lck phosphorylation using an antibody that recognises phosphoserine in the context of an ERK consensus substrate sequence.

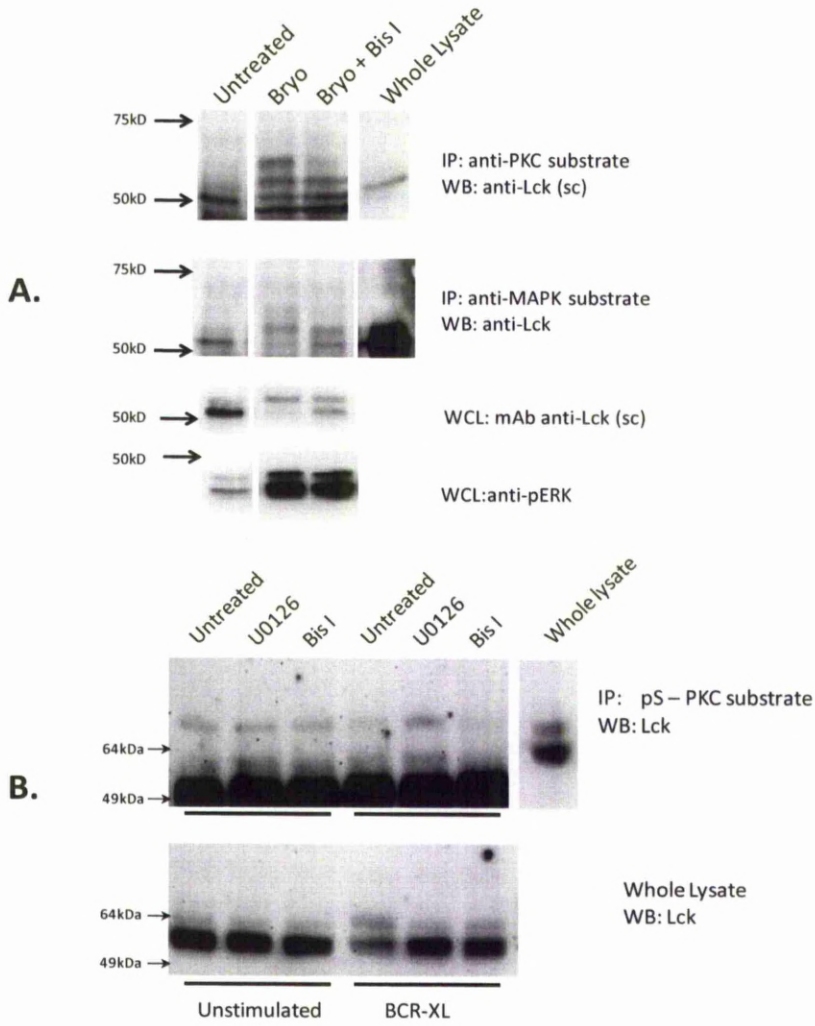


Figure 2.10. Lck from bryostatin-treated CLL cells is a substrate of PKC

A. 1 CLL case incubated with 50nM bryostatin in the presence or absence of 1μM bidindolylamalemide for 1 hour. Cells were pelleted and subjected to immunoprecipitation (IP) with anti-PKC or anti-MAPK substrate antibodies, then western blotted for Lck to identify Lck phosphorylated on serine by PKC or ERK. Whole cell lysates (WCL) were subjected to western blotting for Lck or pERK. **B.** As for A but cells were stimulated with 10μg/ml of $F(ab')_2$ anti-IgM for 1hour in the presence or absence of 10μM of U0126 or 10μM BisI and then subjected to immunoprecipitation of whole cell lysates examined for Lck. Results are representative of two experiments.

Thus, activation of CLL cells with bryostatin resulted in the generation of an anti-Lck reactive band that could be immunoprecipitated with an anti-MAPK substrate antibody. Treatment of CLL cells with 1μM bisI prior to bryostatin stimulation did not affect this immunoprecipitation, likely because the presence of 1μM bisindolylmaleimideI did not affect pERK levels (figure 2.10A).

Finally, I investigated the mechanism of BCR-induced p60 Lck formation. Figure 2.10 shows that the anti-PKC substrate antibody could immunoprecipitate an anti-Lck-reactive protein when CLL cells had been stimulated through the BCR. The results generated with the anti-MAPK substrate antibody were less clear.

Taken together, the results from the above section demonstrate that the appearance of the p60 Lck band is associated with serine phosphorylation of Lck. Moreover, the results also strongly suggest that PKCs and ERK are likely to be involved in mediating this phosphorylation of Lck.

2.3.4.3. Formation of the p60 Lck band is potentially also associated with ubiquitinylation of Lck.

In T cells formation of the p60 Lck band is associated with ubiquitinylation of Lck [96]. I therefore investigated whether this process could also be taking place in stimulated CLL cells (figure 2.11).

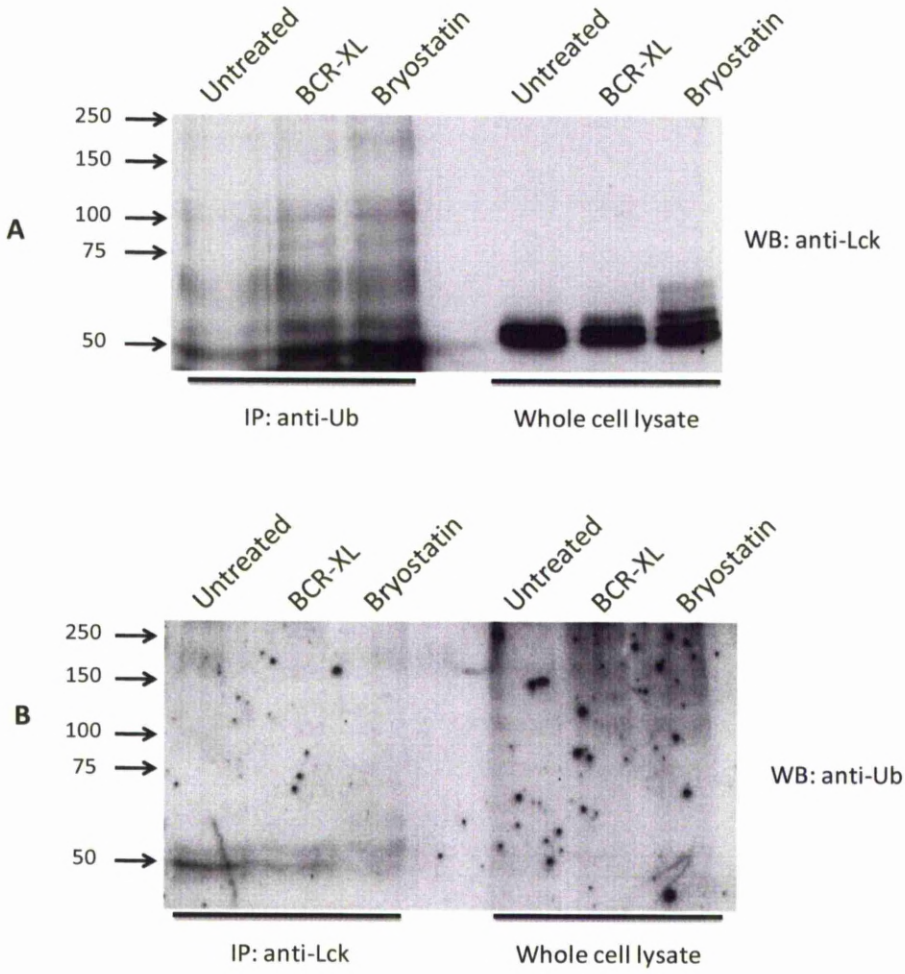


Figure 2.11. BCR XLing induces an increase in smears that react with a ubiquitin antibody from Lck immunoprecipitates. 1 CLL clone was incubated with 10μg/ml F(ab')$_2$ anti-IgM (BCR-XL) or 50nM bryostatin for 1 hour. Ubiquitinylated Lck was assessed either by immunoprecipitating with an anti-ubiquitin antibody and probing for Lck (**A**), or immunoprecipitating with an anti-Lck antibody and Western blotting with the anti-ubiquitin antibody (**B**). Results are representative of three experiments performed on two CLL cases.

Figure 2.11.A shows that anti-Lck reactive proteins could be immunoprecipitated from lysates of BCR and bryostatin-stimulated CLL cells using an antibody that recognises ubiquitin. Several of the reactive bands migrated with the same mobility as the p60 and higher molecular weight Lck bands observed in whole cell lysates. When I did the converse experiment and immunoprecipitated Lck from CLL cell lysates and performed Western blots with the anti-ubiquitin antibody, I found weaker reactivity and the amount of smears indicating polyubiquitintylation of proteins was low even in the whole cell lysates (Figure 2.11B). However, BCR crosslinking or bryostatin stimulation resulted in the appearance of bands in the anti-Lck immunoprecipitates that were reactive with ubiquitin antibodies in Western blots. These bands showed a mobility that was similar to that of the p60 Lck band. Analysis of whole cell lysates with the ubiquitin antibody showed the appearance of high molecular weight smears within the stimulated cell lysates, and this is indicative of ubiquitinylated proteins [96]. Taken together, these results suggest that CLL cells, like T cells, are likely also to ubiquitinylate Lck in response to stimulation.

2.3.4.4. Kinetics of Lck phosphorylation during prolonged CLL incubation with bryostatin

Ubiquitinylation of proteins is often associated with degradation of these modified proteins within the proteasome. A role for ubiquitinylation in the

degradation of Lck in stimulated T cells has already been demonstrated [96]. Since our data suggest that Lck was being ubiquitinylated during BCR and bryostatin stimulation of CLL cells, it was important to investigate whether this led to Lck degradation within these cells.

I initially investigated Lck degradation following activation and the reappearance of the enzyme by Western blot analysis. Figure 2.12 shows that addition of bryostatin to the cells caused an immediate conversion of a part of p56 Lck to the p60 form. In the upper case in the figure (CLL15), this conversion was almost complete during the first 4 hours of stimulation so that the residual p56 band became almost invisible. After this point, the density of the p60 band was progressively reduced in parallel with the progressive reappearance of the p56 band at 4, 24 and 48 hours. At 72 hours, the 60kDa Lck was no longer present and the density of the p56 band had largely recovered and by 120 hours this band even exceeded the level seen in the control unstimulated CLL cell samples.

In the lower case in 2.12A (CLL16), the conversion of p56 Lck to p60 form followed a similar pattern as in CLL15. However the recovery of the p56 band was slower than in CLL15 and the p60 band was still present at later stages of the incubation with bryostatin. Bryostatin activates PKC whereby activated PKC becomes ultimately degraded by proteasomal degradation. Judging from the difference in kinetics of the appearance and

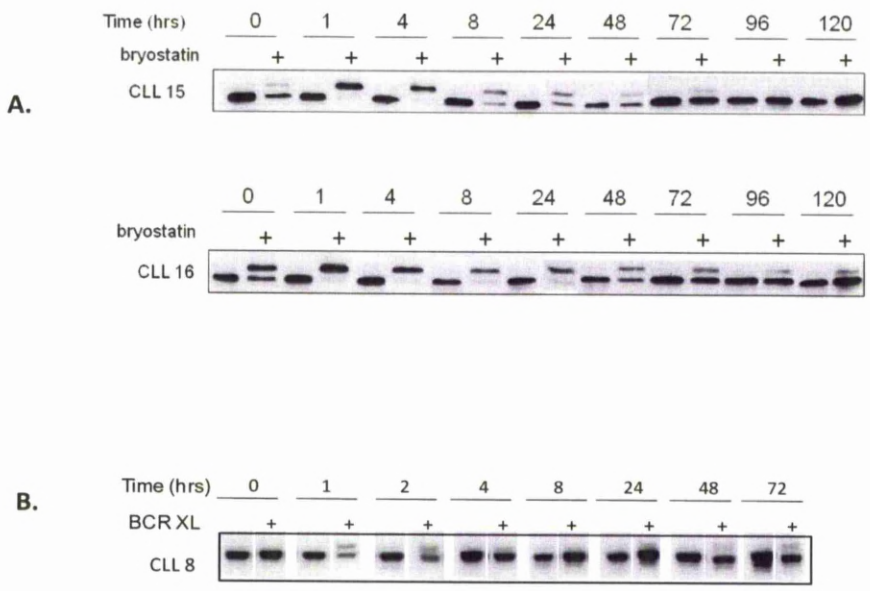


Figure 2.12. Bryostatin or F(ab')$_2$ anti-IgM causes a change in electrophoretic mobility and amount of Lck during prolonged incubation of CLL cells. **A.** cells from 2 CLL patients (CLL15 and CLL16) were incubated with bryostatin at a final concentration of 25nM for 120 hours and examined at indicated times for Lck expression by Western blotting. Results are represenative of two experiments on a total of four cases. **B.** CLL 8 was stimulated with 10μg/ml anti-IgM for the indicated times and analysed for Lck SDS-PAGE migration as in A. Time 0 denotes the time taken to pellet, wash and lyse the cells after the addition of bryostatin or anti-IgM. Representative of two repeat experiments.

disappearance of p56 and p60 bands in clone CLL15 and CLL16 it would appear that PKC activity persisted in CLL16 for a considerably longer time than in clone CLL15.I also examined the kinetics of p60 Lck formation in BCR-stimulated CLL cells. Figure 2.12B shows that stimulation of CLL cells with BCR crosslinking resulted in the generation of the p60 band. The presence of this band was apparent at 1 and 2 hours, but then decreased at 4h, almost disappeared at 8h, but then reappeared at 24h, where it remained for the rest of the experiment. Interestingly, and in contrast to the results observed with bryostatin, the level of the p56 band of Lck decreased in the BCR-stimulated CLL cells at the 48h and 72h time points. Also, BCR crosslinking never stimulated the complete disappearance of the p56 Lck band, whereas bryostatin (or PMA) almost always did.

The above induced changes in Lck mobility could either be due to ubiquitinylation, degradation and *de novo* synthesis of Lck, or to a cycle of protein phosphorylation and de-phosphorylation. If the observed kinetics of appearance and disappearance of the two bands indicates Lck degradation and resynthesis, then the rapid protein recovery indicates *de novo* synthesis through stimulation of Lck translation rather than transcription. This resynthesis also indicates that bryostatin-activated PKC may not only be involved in the regulation of Lck expression through phosphorylation and degradation, but also through regulation of Lck synthesis.

In order to investigate if the p60 Lck band is proteasomally degraded upon bryostatin treatment, the proteasome inhibitor lactacystin was used. CLL cells are particularly susceptible to lactacystin-induced apoptosis [176] and I found that this was the case for most of the CLL samples I used in my studies. However, I found two cases that did not undergo quite as dramatic an induction of apoptosis and would, therefore, allow an experiment investigating the effects of lactacystin on the p60 Lck band to go forward (figure 2.13). Since bryostatin induces PKC downregulation through proteosomal degradation, the treatment of CLL cells with lactacystin will also preserve PKC levels within the treated cells. This persistent presence of active PKC would likely contribute to Lck phosphorylation, and consequently cause an increase in the level of the p60 Lck band independent of any effect on Lck degradation. To eliminate this problem I included bisindolylmaleimideI in my experiments as a general PKC inhibitor.

Figure 2.13A shows that 24 and 48 hours incubation with bryostatin produced the expected shift of the p56 Lck band to the p60 position. This accumulation of the p60 Lck band was almost completely abrogated by the addition of lactacystin in the two CLL cases shown. This disappearance of the p60 Lck band induced by lactacystin is unlikely to be due to the cytotoxicity of this reagent on CLL cells because this reagent reduced CLL cell viability by approximately 50% in the two cases that I used, and

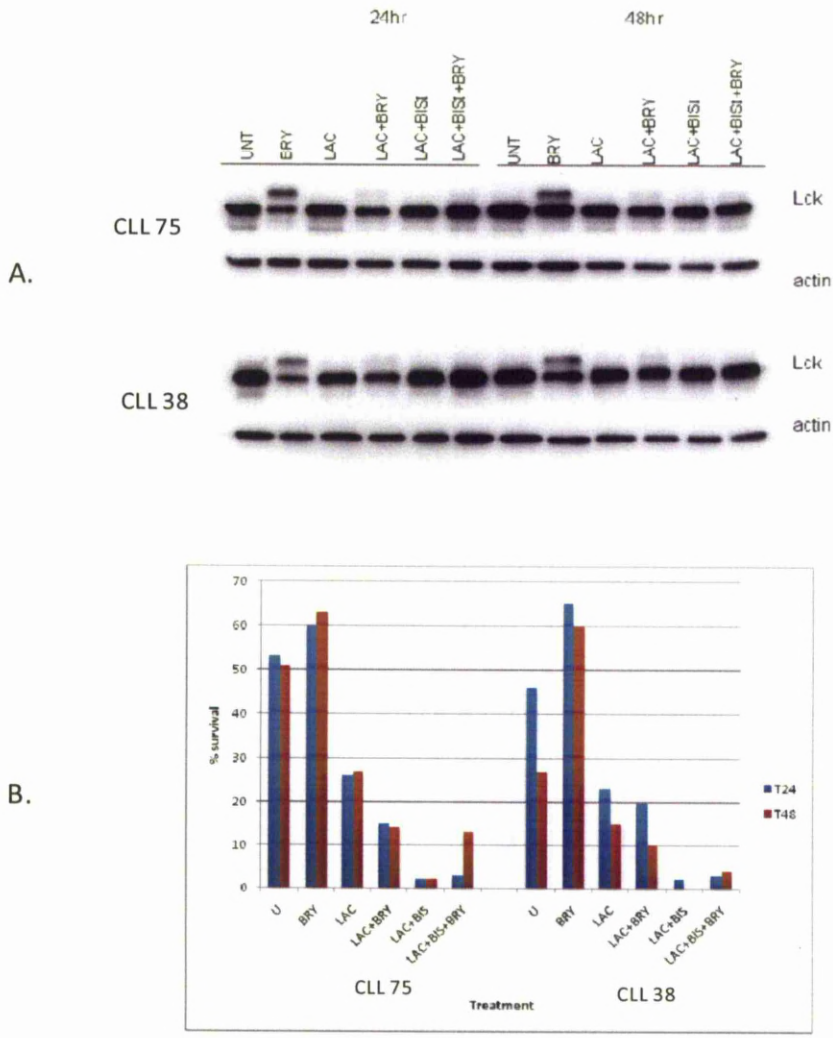


Figure 2.13 Lactacystin treatment decreases the bryostatin-induced formation of p60lck. 2 CLL cases were pre-incubated with 2.5μM lactacystin or 10μM bisindolylmaleimideI for 1 hour before addition of 25nM bryostatin to the relevant wells. **A;** Cells were pelleted, lysed in clear SDS sample buffer, protein determination carried out and 10μg total protein subjected to Western blotting for Lck and then the blot reprobed with an anti β-actin antibody as a loading control. **B;** apoptosis assay showing percentage alive cells (DiOC6 positive, PI negative) after 24 or 48 hours incubation with 25nM bryostatin (BRY), 2.5μM lactacystin (LAC) and 10μM bisindolylmaleimideI (BISI). Results represent one experiment.

because this reduction in cell viability is not matched by a proportional decrease in p60 Lck band density. Since lactacystin treatment did not result in maintenance of the p60 Lck band but instead fostered the appearance of the p56 Lck band, there is a suggestion that the appearance of the p60 Lck band resulting from bryostatin treatment does not necessarily lead to proteomic degradation of Lck.

The inclusion of bisindolylmaleimideI in these experiments reduced CLL cell viability to near zero levels. Therefore, the effect of BisI and lactacystin on Lck mobility cannot be interpreted.

An alternative method for showing the fate of the p60 Lck band would be to treat the cells with siRNA targeting Lck. Knockdown of Lck mRNA would mean that if p56lck were to reappear after the shift to p60lck then this could not be due to newly synthesised protein and must therefore be due to dephosphorylation of p60lck. However, although I tried this procedure several times, using techniques such as nucleofection or reagent-mediated transfer, this did not result in a decrease in Lck protein levels. To show that my method was working a FITC-labelled siRNA was used to confirm the uptake of siRNA into the cells. Figure 2.14C shows that CLL cells incorporated the FITC-siRNA. Figure 2.14A shows that despite using two different siRNA (from Ambion and Qiagen) specifically designed to knock down Lck mRNA, there was no change in Lck protein expression,

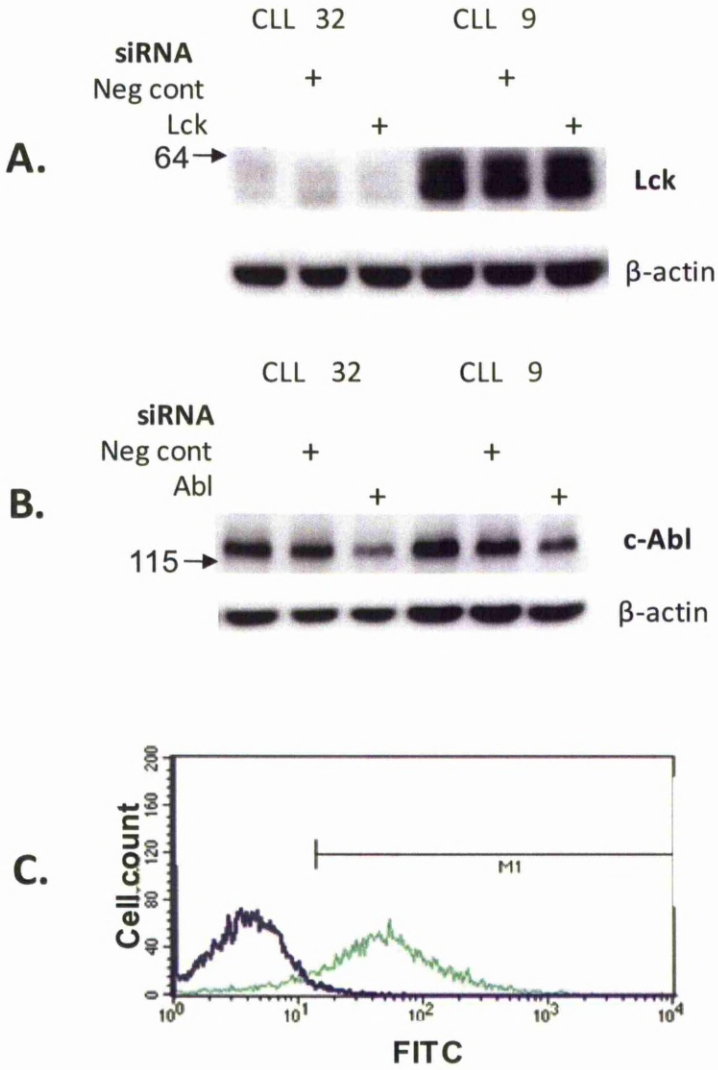


Figure 2.14. Lck and c-Abl targeted siRNA treatment of two CLL cases. Cells were subjected to 2 hits of siRNA at a concentration of 100nM for a total of 96 hours. Amount of Lck **(A)** or Abl protein **(B)** following siRNA treatment of two CLL clones. **C.** FACS plot showing uptake of a positive control FITC-labelled siRNA(■) in >85% of cells in comparison to the untransfected cells (■). Representative of two experiments.

even if CLL cells were incubated for up to 96h. Similar results were generated using another siRNA (a mixture of 4 different siRNA in a preparation called Smartpool™ siRNA from Dharmacon). In contrast, siRNA targeting c-Abl resulted in a small decrease in c-Abl protein expression (Figure 2.14B), and replicates the results of experiments already performed in this department [177]. This persistence of Lck protein in the cells after siRNA treatment is presumably due to a very slow Lck protein turnover in CLL cells that have been deprived of external stimuli, or to an inability of the Lck siRNA to properly reduce Lck mRNA levels.

Therefore, inhibitors of transcription and translation were used to investigate the possibility that the p56 Lck band is newly synthesised protein. I used two inhibitors of gene transcription, actinomycin D and flavopiridol, and one inhibitor of protein translation, cycloheximide. Like lactacystin, these reagents are very cytotoxic to CLL cells [178-182]. Ideally, these inhibitors should have been used in 24 - 48 hour incubations in the presence of bryostatin, but, in order to limit the effect of these inhibitors on CLL cell viability, relatively short incubation times were used (4 and 12 hours).

Figure 2.15 shows that flavopiridol and cycloheximide were active in CLL cells and resulted in the downregulation of Mcl1 expression. This result was expected based on the reliance of Mcl1 on new transcription and

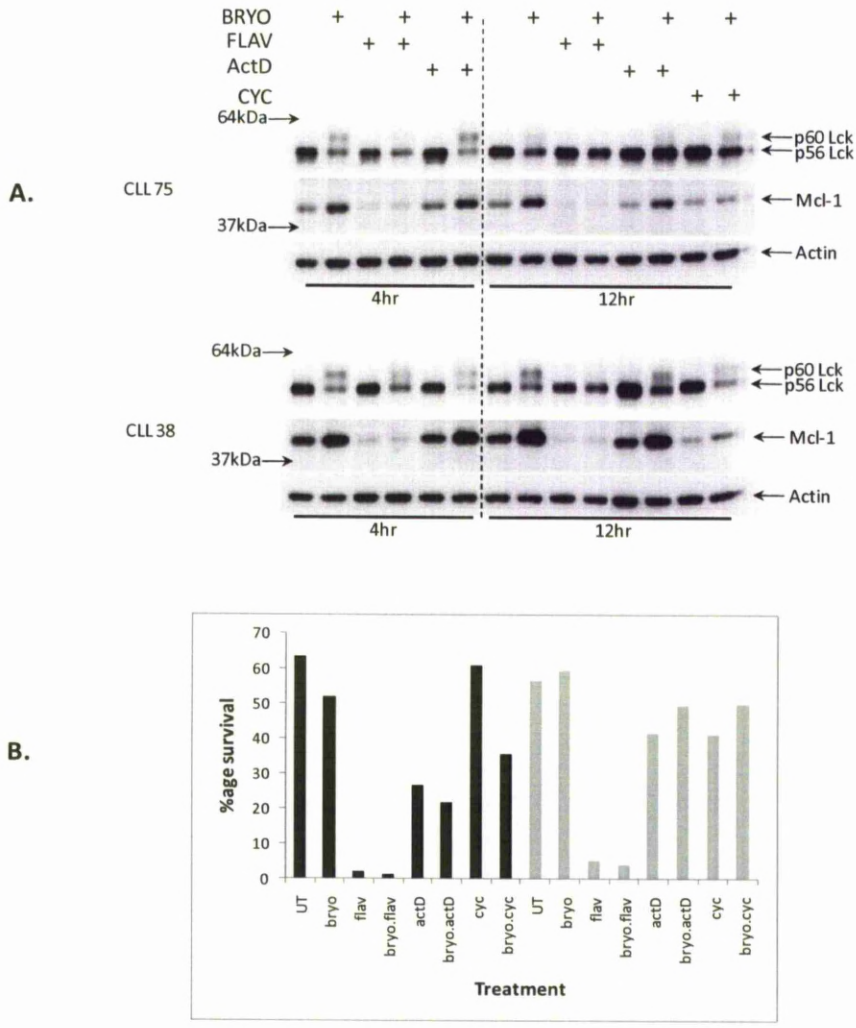


Figure 2.15 Use of transcription and translation inhibitors to investigate the fate of the p60 band of Lck. 2 CLL cases were incubated with or without 25nM bryostatin (BRYO), 2μM flavopiridol (FLAV) or 8nM of actinomycin D (ActD) for 4 hours. At the 4 hour timepoint 10μM cycloheximide (CYC) was added and the culture was continued for a further 8 hours. **A.** Western blotting for Lck, Mcl-1 as a positive control for the action of flavopiridol or β-actin as a loading control. **B.** The effect of the different inhibitors on CLL cell survival after 12 hours (CLL 75 black bars, CLL 38 grey bars) of incubation was assessed by a $DiOC_6$/PI apoptosis assay. Representative of two experiments.

translation in order to maintain its expression levels, and confirms previously published results [183]. Actinomycin D seemed only to work in the upper CLL case but not in the lower one.

With respect to Lck, incubation of bryostatin-treated CLL cells with flavopiridol and cycloheximide resulted in a decrease in intensity of the p60 band and reappearance of the p56 band. The effect I observed with respect to flavopiridol at 4h is not due to the cytotoxicity of this reagent because its removal from CLL cultures at this time point is reported to reverse the events leading to the induction of apoptosis [184]. At the 12h time point flavopiridol had a considerable cytotoxic effect (Figure 2.15), so the effects of this agent on Lck cannot be interpreted. Nevertheless, the disappearance of the p60 Lck band and the strong presence of p56 Lck band at the 4h time point is reminiscent of the results generated with lactacystin. The fact that flavopiridol induced strong downregulation of Mcl1 but did not largely affect Lck suggests that Lck protein is turned over at a much slower rate in CLL cells than is Mcl1, and that stimulation of the cells with bryostatin does not change this turnover.

However, with cycloheximide the disappearance of the p60 Lck band did not correspond to the same strong reappearance of the p56 Lck band as was observed with flavopiridol at 4h. This result is noteworthy because it is

not associated with induction of significant cell death, and suggests that at least some protein synthesis contributes to the reappearance of p56 band.

Taken together, the results of this section suggest that the appearance of the p56 Lck band in CLL cells following bryostatin treatment is partially due to *de-novo* protein synthesis. The disappearance of the p60 Lck band may not be totally due to proteosomal degradation because lactacystin failed to maintain the presence of this form of Lck. Thus, p60 is likely also to be a target of protein phosphatases, whereby p56 is generated through the dephosphorylation of p60.

2.3.4 Expression of Lck protein is differentially affected by BCR crosslinking and IL-2 stimulation in different CLL cell clones

Lck expression can be induced in normal B cells through stimulation of these cells with BCR crosslinking and IL-2 [113]. To examine whether the same mechanisms also operated in CLL cells, 4 different CLL cell clones and normal peripheral blood B cells were incubated for 72 hours with either anti-IgM or IL-2 or a combination of both stimuli. The results of this experiment are shown in figure 2.16.

Thus, in agreement with Taieb *et al*'s results [113], combined IL2 stimulation and BCR crosslinking for 72 hours increases Lck protein expression in normal B cells. However, it should be noted that IL-2 alone

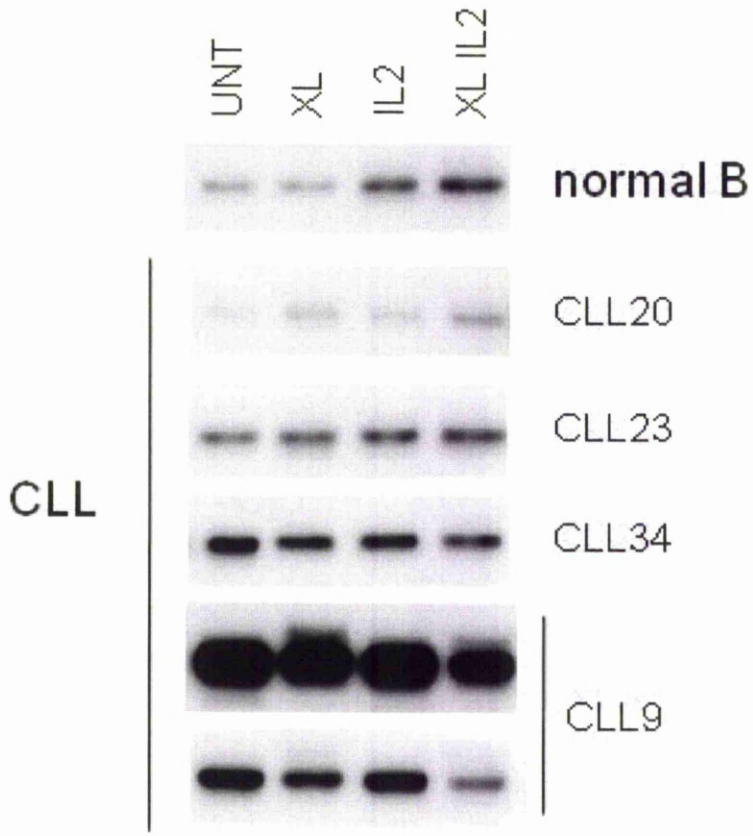


Figure 2.16. IL-2 and BCR stimulation do not up-regulate Lck protein in CLL cells. Normal peripheral blood B cells or the cells from 4 CLL cases were cultured with 10μg/ml anti-IgM, 100IU/ml IL-2 or both for 72hrs. Lysates were subjected to Western blotting with an anti-Lck antibody. The darkness of the Lck bands in case CLL9 with very high protein expression was reduced using computer image software (AIDA 2D densitometry) to show the decrease in protein upon BCR stimulation that was not clear in the upper, original image, due to overexposure. Result is representative of two experiments.

was sufficient to induce Lck protein, and that combination of IL-2 and anti-IgM had only a small additional effect. A similar result showing that IL-2 stimulation of T cells induces an upregulation of Lck protein levels has been reported [185].

In contrast to the results generated for normal B cells, the pattern of CLL cell response to these stimuli varied from case to case, and was different from the response of normal B cells. If the Lck expression in untreated cell was very low (CLL20) then BCR crosslinking alone induced a small increase in Lck. In CLL23 and CLL34 that express intermediate levels of Lck in unstimulated control cells, the incubation of cells with any of above stimuli alone or in combination had no clear effect on Lck expression. However in CLL9, which had a very high level of Lck, the amount of Lck protein was reduced rather than increased when the BCR was cross-linked by anti-IgM, and the degree of this reduction was strongly potentiated in cells stimulated by the combination of anti-IgM and IL-2.

It would appear therefore that, depending on the level of Lck expression, CLL cells either do not respond to the stimuli that increase the expression of this protein in normal B cells or, contrary to what is seen with normal B cells, the high levels of Lck present in some CLL cells is actually reduced upon cell stimulation by anti-IgM and IL2. This decrease in Lck upon BCR cross-linking could be due both to activation of PKC and/or ERK, which

are shown to phosphorylate Lck, and to its ubiquitinylation which would direct it to proteasomal degradation.

2.4 Discussion

The presence of Lck mRNA in CLL cells was shown by others [4, 128], and now confirmed in the present study (figure 2.1a and 2.1b). Moreover, the present study was the first attempt to characterise all possible transcripts in CLL cells that are generated from both the proximal (type I) and distal (type II) promoter.

The results showed that, like some other lymphoid cells of malignant origin [115], CLL cells contain both type I and type II Lck mRNA transcripts. The more dominant type II transcript is in normal mature lymphocytes associated with antigen receptor signalling [3]. Therefore the finding of this transcript in CLL cells supports the published data showing that these cells are mature, antigen-activated cells. In addition, CLL cells contain variable levels of type IA and B transcripts which are normally associated with cells undergoing malignant transformation. The IIA and IB transcripts that were amplified by the primers I used are the correct size for the expected products. However, having analysed the complete sequence of the Lck gene on the Entrez Nucleotide database it would appear that the IA and pre-IA products as assigned by Rouer et al [115], may represent a sequence containing introns due to either contamination with DNA or the

presence of a mature unspliced version of mRNA which would possibly not be translated into a functional protein.

Although there was some difference in the expression of different transcripts between different CLL cases, the PCR method used was not sufficiently quantitative to correlate Lck mRNA expression with other phenotypic and functional features of CLL cells. Ideally, the levels of the different transcripts would require analysis by quantitative real-time PCR (qRT-PCR). However, the multiple sizes of products formed from the amplification of the transcripts present in CLL cells would give a number of peaks which makes qRT-PCR unsuitable for quantitative analysis of Lck transcript expression from the different promoters.

A different set of primers were designed to amplify a sequence in the coding regions of both transcript 1 and 2 which could be used for qRT-PCR and therefore would allow for quantification of Lck mRNA. CLL cases analysed were selected based on Lck protein expression as assessed by western blotting (figure 2.3). Normal T and B cells and one representative HCL sample, that contained very little Lck protein, were also analysed. CLL cells, even those that contain very low levels of Lck protein, contain levels of Lck mRNA that are similar to the levels seen in normal T cells. When I compared Lck mRNA with protein levels I found that these two parameters were not correlated in the CLL cases I analysed

(R^2=0.033). That is, CLL cells from cases expressing high levels of Lck protein had levels of Lck mRNA which were similar to those expressed in CLL cells from cases expressing low levels of Lck protein. This result suggests that the turnover of Lck protein is likely to be very slow in CLL cells that allows for a steady build up over time.

The data presented in this chapter confirm published work showing Lck protein expression in normal and malignant B cells [4, 126]. In most cases, expression of Lck in these cells will depend upon the stage of maturation or the degree of activation [3]. With respect to CLL cells, these may vary with respect to either maturation or activation, or both, so it can be expected that differences in the level of Lck expression between different clones will be observed. I used highly purified CLL cells in my analysis to confirm the presence of Lck, and further show that Lck expression is widely variable between CLL cell clones. It is unlikely, however, that levels of Lck protein have a prognostic value because they did not correlate with established markers of disease prognosis such as IgVH mutation and ZAP-70 expression.

The malignant HCs shown in figure 2.4 did not contain Lck protein although they had detectable Lck mRNA (figure 2.1 and 2.2). Also, other HCL cases tested during the course of this study (n=6) either completely lacked Lck or contain very low levels of this protein. Since HCs are

arrested at closely similar maturation stage as CLL cells [186, 187], the absence of Lck in these cells made them an ideal negative control for the subsequent studies of the role of Lck in CLL signalling and survival. In contrast to HCL cells, the expression of Lck in MCL was variable (n=5, 1 representative case shown in figure 2.3) confirming recently published data [108]. 4 of the MCL cases had a very low level of Lck protein, but one case shown in figure 2.3, had moderate levels of this kinase. This level was comparable to the amount of Lck present in some CLL clones.

In addition to regulation by transcription and translation, the levels of Lck protein in T cells are also known to be controlled post-translationally by activation, followed by ubiquitination and proteasomal degradation. The activation of Lck in CLL cells, as assessed by migration in SDS-PAGE gels to the 60kDa level, was observed in the present study in anti-IgM treated cells and cells treated by the PKC activators PMA and bryostatin. In this respect, CLL cells resemble T cells in their ability to modify the mobility of Lck on Western blots. We further show that CLL cells induce formation of the p60 Lck band through serine phosphorylation that is likely catalysed by activation of PKC and of ERK. This was demonstrated by the use of specific inhibitors of PKC and MEK; these inhibitors blocked the formation of the p60 Lck band that was induced in CLL cell stimulated by BCR crosslinking and PMA/bryostatin. Moreover, we also demonstrate that Lck can be immunoprecipitated by antibodies that recognise phospho-

serine within the consensus recognition sequences of PKC and ERK. Serine 42 and 59 of Lck are contained within such recognition sequences [97], suggesting that our ability to immunoprecipitate Lck with these antibodies is due to recognition of serine phosphorylated Lck and not to co-immunoprecipitation of Lck with other serine-phosphorylated proteins. Our ability to directly immunoprecipitate Lck with PKC- and ERK-substrate specific phospho-serine antibodies is also demonstrated by the molecular weight of the immunoprecipitated Lck, which had a mobility corresponding to that of the p60 Lck band.

I also show that Lck can be ubiquitinylated in CLL cells. Ubiquitinylation of proteins is an important process whereby they are tagged for proteosomal degradation, and/or for targeting the protein into another cellular compartment. Polyubiquitinylation normally results in proteosomal degradation of proteins, whereas monoubiquitinylation can result in the use of a particular molecule for further signalling. In T cells, TCR engagement induces polyubiquitinylation and proteosomal degradation of Lck [96]. In my experiments I was unable to show that Lck was degraded within the proteosome of CLL cells due to the high cytotoxicity of lactacystin. However, the results of the experiments using flavopiridol (to inhibit protein transcription) and cycloheximide (to inhibit protein translation) suggest that Lck protein in CLL cells is degraded at a far slower rate than

is Mcl1, and that this rate of degradation is not affected by formation of the p60 band.

Taken together, the results of this Chapter demonstrate the CLL cells express Lck mRNA and protein. The data in this Chapter also show that Lck can be post-translationally modified by PKC and ERK in CLL cells undergoing activation stimuli, but that this modification does not affect the rate at which Lck is degraded in the cell.

Chapter 3

The role of Lck in CLL cell cytoprotection

3.1 Introduction

The function of Lck in normal B cells and CLL cells has so far not been investigated in depth, mainly because of the long lasting uncertainty whether this enzyme is expressed in these cells at all. In addition, many functional responses which are in T cells regulated by Lck are in B cells dependent on Lyn. Thus, in normal B cells as well as in CLL cells the focus so far was on Lyn as the principal SFK that initiates signalling responsible for antigen-mediated selection, maturation and clonal expansion of these cells.

Having shown that the comparatively high levels of Lck protein present in CLL samples originates from malignant CLL cells and not from contaminating T cells, the next step was to investigate whether this enzyme has any non-redundant, specific functional role in CLL cells not shared by other SFKs. Because the most important factors responsible for clonal expansion of CLL cells are those involved in cell proliferation and survival, and since CLL cells do not proliferate *in vitro*, it was decided to

examine first whether specific inhibition of Lck affects CLL cell viability in *in vitro* cultures.

3.2 Materials and methods

3.2.1 Cell isolation and culture

CLL and HCL samples were obtained and cultured as described in methods section 2.2.1

3.2.2 Inhibitors and stimuli

All inhibitors were dissolved in DMSO, aliquoted and stored at -20°C. PP1 (Biosource; Oxford Biosystems, Wheatley, UK) was always used at 10μM. The Lck inhibitor (4-Amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2-d]pyrimidin-7-yl-cyclopentane) was used at 1μM, , lactacystin was used at 5μM, RO-32-432 was used at 10μM and bisindolylmaleimide I was used at either 1μM or 10μM, and all were purchased from Calbiochem. The caspase inhibitor (Z-Val-DL-Ap-fluoromethylketone) which was used at 500nM was purchased from Bachem (St Helens, UK).

$F(ab')_2$ fragments of goat anti–human IgM were used at a final concentration of 10μg/ml for crosslinking/stimulating the BCR (Jackson ImmunoResearch Laboratories). CpG stimulation utilised 0.5 or 5μg/ml ODN2006 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') and ODN2006 control (5'-TGCTGCTTTTGTGCTTTTGTGCTT-3') oligonucleotides (InvivoGen; Autogen Bioclear, Calne, UK) for human Toll-like receptor 9 (TLR9) stimulation. CD40 ligand (CD40L) was supplied with an enhancer

in a soluble human recombinant set from Alexis biochemicals. The CD40L was incubated with the enhancer in cell culture media at 37°C for 30 minutes and then added to the cells at a final concentration of 200ng/ml ligand and 1μg/ml enhancer.

3.2.3 Western blotting

As described in 2.2.3. In addition, blots were blocked in 5% milk dissolved in TBST for analysing the expression of proteins, or 5% BSA in TBST for analysis of phosphorylated proteins. Antibodies were prepared in TBST containing either 5% milk powder or 5% BSA as for blocking of blots.

The following primary antibodies were used: anti-Lck mouse monoclonal, anti-pERK mouse monoclonal, anti ERK rabbit polyclonal and anti-Mcl-1 rabbit polyclonal (Santa Cruz Biotechnology). Anti-pY^{416}Src, anti-$pSer^{473}$Akt mouse monoclonal, anti Bcl-xL rabbit polyclonal, anti-c-IAP1 rabbit polyclonal antibody, anti-cIAP2 rabbit polyclonal antibody, anti-pIKK rabbit polyclonal, anti-PARP mouse monoclonal, anti-BIM rabbit polyclonal and anti-XIAP rabbit polyclonal antibodies were purchased from Cell Signaling Technology. Anti-p21WAF1 (clone DCS-60.2) mouse monoclonal antibody was obtained from Biosource. Anti p27 antibody was purchased from BD transduction laboratories. The following secondary antibodies were used: Goat anti-rabbit horseradish peroxidise

(HRP) conjugated antibody, goat anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology).

3.2.5 Immunoprecipitation

$1x10^7$ cells in media were pelleted at 500g for 5 minutes at 4°C and then lysed in 1ml of 4°C RIPA lysis buffer (PBS, 20mM Tris, 316mM NaCl, 2mM $EGTANa_2$, 2% TX-100, 0.02% SDS) containing phosphatase and protease inhibitors (1mM PMSF, protease inhibitor cocktail set III-EDTA free (Calbiochem) 1:100 dilution to produce a final concentration of 1mM AEBSF, 800nM aprotinin, 50μM bestatin, 15μM E-64 protease inhibitor, 20μM leupeptin and 10μM pepstatin A). Lysates were then incubated for 15 minutes on ice to allow complete lysis of the cells. Lysates were then centrifuged at 18000g for 10 minutes to pellet unlysed material. 900μl of supernatent was transferred to a new eppendorph and then incubated overnight at 4°C with 5μg of immunoprecipitating antibody, followed by 1 hour incubation with 30μl protein A/G beads (Invitrogen). Protein-A/G immuno-complexes were pelleted by centrifugation at 18000g for 10 minutes, then washed 3 times with 4°C RIPA lysis buffer. Samples were boiled in 30μl 2xSDS laemelli sample buffer, run on a polyacrylamide gel and subjected to Western blotting.

3.2.6 Cell survival analysis

1ml of $2x10^6$/ml cells in culture media were incubated with various stimuli in the presence or absence of 1μM of the Lck inhibitor for 48 hours and the relative proportion of alive or dead cells assessed using a 3,3-dihexyloxacarbocyanine iodide/propidium iodide ($DiOC_6$/PI) apoptosis assay.

3.3 Results

In chapter 2, I used siRNA in an attempt to inhibit Lck mRNA and decrease protein expression in CLL cells (figure 2.14). Although I could show that the technique had the potential of working, particularly with proteins such as c-Abl, I found Lck protein expression in CLL cells was not affected by the presence of siRNA. This meant that any assessment of Lck function in CLL cells had to be derived from comparison with HCL-cells serving as negative controls, and on the specificity of a commercially available inhibitor of Lck activity.

3.3.1 Lck in CLL cells is specifically inhibited at 1μM concentration of an inhibitor of Lck activity

Due to the close structural homology of the active site of SFKs it had to be assumed that inhibitors targeting this site are relatively non-specific. It was therefore necessary to confirm that the 4-Amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2-d]pyrimidin-7-yl-cyclopentane, (from now on referred to as the Lck inhibitor) employed in this study, is indeed specific for Lck at the concentrations used.

Figure 3.1 shows the structure of the Lck inhibitor. This compound is reported to have an IC_{50} for Lck that is about 3000 fold lower than for c-Src and 26 fold lower than for Lyn [188]. Nevertheless, particular attention had to be paid to the possibility that this inhibitor also inhibits Lyn, since it

has been shown that this kinase is highly expressed in CLL cells and has an important function in CLL cell survival [132].

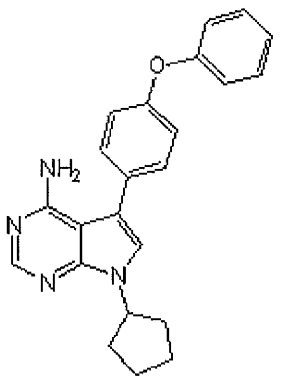


Figure 3.1 Structure of the Lck inhibitor

Figure 3.2 shows the effect of cell incubation with the Lck inhibitor on active site phosphorylation of Lck and Lyn in non-stimulated and BCR-stimulated CLL cells. To demonstrate specificity of these effects, the SFKs were first immunoprecipitated from cell lysates by an antibody which recognises pY416 in the active site of Src (anti-active Src). This antibody also crossreacts with active-site phosphotyrosines in Lck (pY394) and in Lyn (pY396), as well as in other SFKs. The immunoprecipitates were then analysed by Western blot using specific anti-Lck (A) and anti-Lyn (B) antibodies. Thus, the anti-active Src antibody immunoprecipitated Lck as a doublet in unstimulated CLL cells. This shows that Lck is constitutively active in these cells. Stimulation of CLL cells with BCR crosslinking

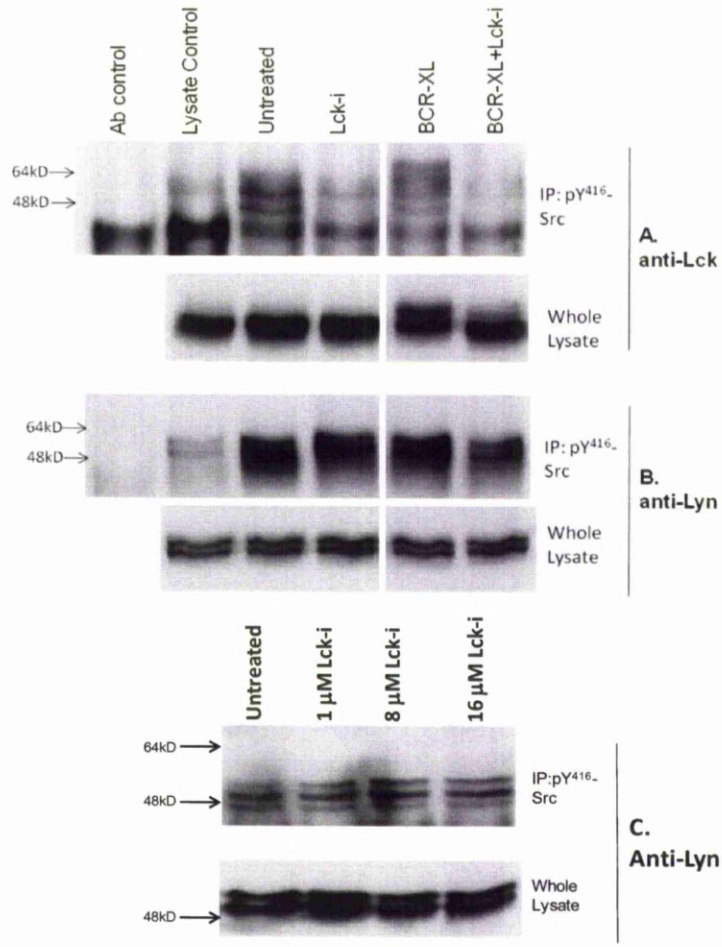


Figure 3.2. The Lck inhibitor primarily inhibits the activity of Lck. CLL cells (CLL9) were pre-incubated with the Lck inhibitor at 1µM for 1 hour and then stimulated with $F(ab')_2$ anti-IgM at 10µg/ml final concentration for 30 minutes. The cells were then washed, pelleted and lysed in a RIPA buffer followed by immunoprecipitation (methods section 3.2.5) by an anti-pY416 antibody which recognises autophosphorylation of the active site specific tyrosine of Src as well as Lck and Lyn. Immunoprecipitates were then subjected to Western blotting for Lck (**A**) or Lyn (**B**). **C**; As for B, but increasing concentrations of the Lck inhibitor (1µM, 8µM or 16µM as in figure 3.2) were used.

resulted in a shift in molecular weight of the anti-Lck bands that were immunoprecipitated with the anti-active Src antibody. This shift in molecular weight corresponds to the appearance of the p60 Lck band in the whole cell lysate and therefore reflects the additional serine phosphorylation of Lck that results in CLL cells from BCR stimulation. Pre-treatment of resting or BCR-stimulated CLL cells with the Lck inhibitor resulted in a strong reduction in the intensity of Lck-reactive bands. This suggests that the Lck inhibitor was blocking Lck activation in CLL cells.

We next examined the Western blots for the presence of Lyn. Thus, the anti-active Src antibody immunoprecipitated copious amounts of Lyn from lysates of resting and BCR-stimulated CLL cells (figure 3.2B). This confirms the results of others relating to the presence of active Lyn in CLL cells [132]. The presence of the Lck-inhibitor had no effect on the ability of the anti-active Src antibody to immunoprecipitate Lyn. These results suggest that the Lck inhibitor specifically acts to block Lck activity in CLL cells. Figure 3.2C shows that even at very high concentrations of the Lck inhibitor, the ability of the anti-active Src antibody to immunoprecipitate Lyn remained largely unaffected. Taken together, these experiments show that the Lck inhibitor I have employed in this study was relatively specific for this kinase, and justified its use for the remainder of this thesis for the investigation of the functional importance of Lck in CLL cells.

3.3.2 Lck contributes to spontaneous CLL cell survival

Having demonstrated specificity of the Lck inhibitor for Lck activity, 4 different CLL cell clones and malignant cells from 2 patients with HCL were treated with this inhibitor at concentrations progressively increasing from 32nM to 16μM. The results are shown in figure 3.3.

Thus, the survival of CLL cells was variably reduced by the Lck inhibitor in a concentration dependent fashion (Figure 3.3). In contrast, the survival of HCL cells was largely unaffected by the presence of the Lck inhibitor, even at the very highest concentration. The malignant cells of HCL express very little or no Lck (figure 2.3), but have been shown to undergo apoptosis in response to the general SFK inhibitor PP1 [189]. These data demonstrate that the Lck inhibitor has no effect on the SFKs that are important for HCL cell survival. When taken together with the data presented in Figure 3.2 showing that the Lck inhibitor does not affect Lyn activity, these data further suggest that the ability of the Lck inhibitor to reduce CLL cell viability is due to its ability to specifically affect Lck, and implicates a role for Lck in CLL cell cytoprotection. Since 50% viability inhibition was observed at around 1μM of the inhibitor, this concentration was chosen for all future experiments.

In order to further explore how effective the Lck inhibitor is at reducing viability of CLL-cell clones belonging to the different prognostic

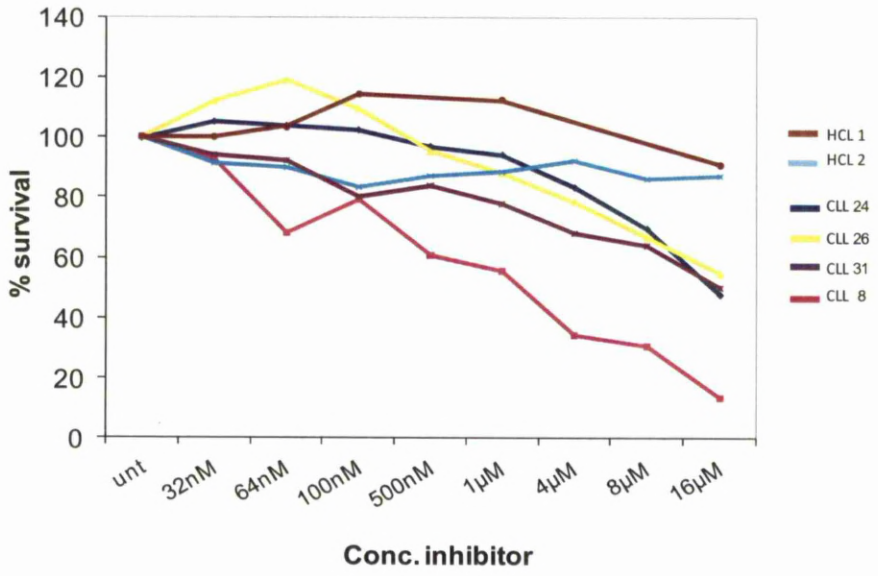


Figure 3.3 The Lck inhibitor variably affects the survival of CLL cells but not HCL cells The effect of the inhibitor on cell survival was analysed by FACS after 48 hour incubation, using the PI/$DiOC_6$ cell viability assay (as detailed in methods section 3.2.6). This technique identifies the proportions of alive ($DiOC_6$ positive and PI negative), early apoptotic ($DiOC_6$ and PI negative) and dead ($DiOC_6$ negative and PI positive) cells within the analysed population. The proportion of live cells at the end of the 48 hour incubation with the Lck inhibitor was expressed as a percentage difference between inhibitor-treated and untreated control cells, assigning the value of 100% viability to the latter cells.

subgroups, 56 CLL-cell samples were incubated with 1μM of the inhibitor. Again, three different HCL cell clones, which contain other SFKs but little or no Lck protein, were used as negative controls to confirm the specificity of the Lck inhibitor. Figure 3.4A shows that there was a wide variation in the effect of the Lck inhibitor on the survival of different CLL clones, ranging from 80% decrease in cell survival to no effect at all. In contrast, the presence of the Lck inhibitor, as expected, had largely no effect on HCL cell survival. Among the 56 CLL cases analysed, 27 were IgVH gene-mutated and 29 were unmutated. When I analysed CLL cell sensitivity to the Lck inhibitor in relation to IgVH status (figure 3.4B), I found that the malignant cells from unmutated CLL cases were generally more sensitive to the Lck inhibitor than were those from mutated CLL cases (P=0.04). Since IgVH mutation is a good predictor of disease progression in CLL, these data suggest that the role Lck plays in CLL cell cytoprotection may have more significant consequences in those cases that are likely to progress. This notion is supported by previous observations from this department [190] showing that Lck activity measured by an *in vitro* kinase assay is higher in the malignant cells from unmutated CLL cases than in those from mutated CLL cases, and that BCR signalling and ERK activation (shown in chapter 4 to involve Lck) was selectively important for cytoprotection of unmutated and not mutated cells. However, the degree of induction of apoptosis in response to the Lck inhibitor in the current study did not correlate with the amount of Lck protein present in

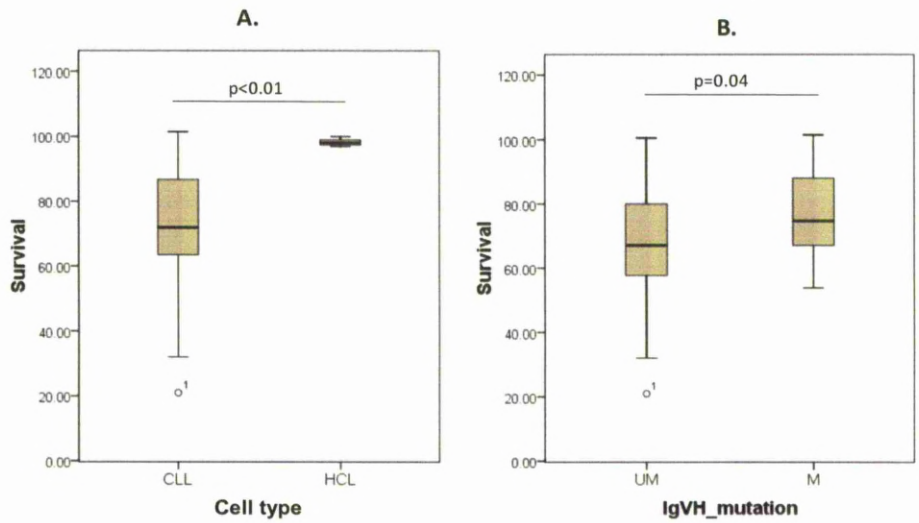


Figure 3.4. The Lck inhibitor variably decreases spontaneous survival of CLL cells. Unmutated CLL cells are affected to a greater degree than mutated cells Cells from 56 CLL and 3 HCL cases were incubated for 48hours in the presence of 1μM Lck inhibitor. Cell viability was assessed using a $DiOC_6$/PI apoptosis assay and is expressed as a percentage survival of treated relative to untreated cells. Cases with CLL-cell IgVH mutation below 2% are designated unmutated (UM) and those with 2% or above are designated mutated (M). Statistical significance was assessed by an unpaired T-test and the p value is displayed on each boxplot. Point 1 on the boxplots represents an outlier (with 21% survival). Results are the mean of two experiments.

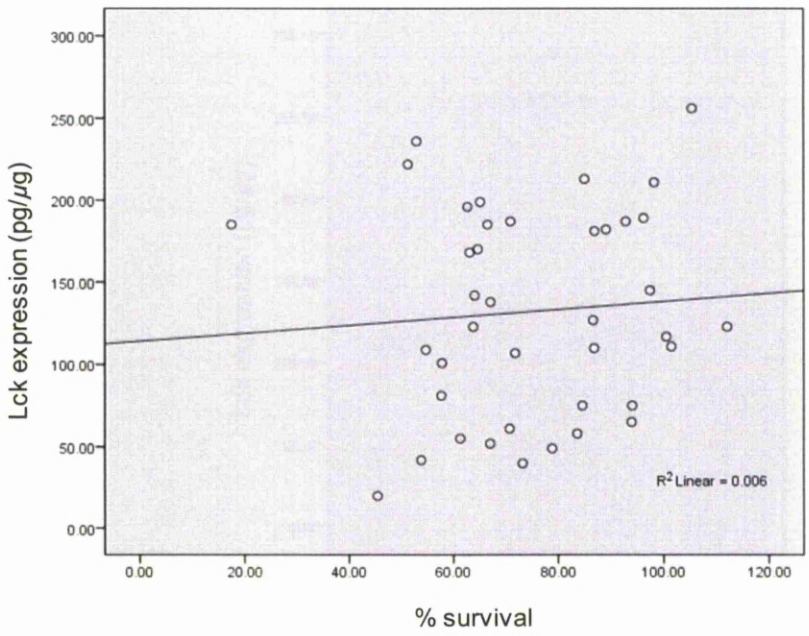


Figure 3.5. The degree of Lck inhibitor-induced apoptosis in CLL clones does not correlate with levels of Lck protein. Cell survival was assessed as for figure 3.4 in 42 CLL cases and compared to the level of Lck protein expression in each case. The r^2 value is 0.006 and is therefore not significant. Cell survival data is the mean value of two experiments.

each case (figure 3.5). This indicates that CLL-cell survival depends more on the degree of Lck activation than on the level of Lck protein.

3.3.3 Lck is also involved in cytoprotection of CLL cells following activation by a variety of different stimuli

Having demonstrated that active Lck is important in maintenance of the viability of apparently quiescent CLL cells it seemed important to see whether Lck also has a role in cytoprotection of these cells when exposed to a variety of known CLL activation stimuli.

The results of 48 hour incubation of cells from high and low Lck expressing CLL clones with different stimuli known to activate these cells or protect them from apoptosis are shown in figure 3.6. 1μM Lck inhibitor was employed to assess the role of Lck in providing cytoprotection of these stimulated cells by comparing their viability when stimulated in the presence or absence of this inhibitor. Unstimulated cells were again designated as 100% viable at the end of incubation and used as a control to measure the changes in the viability of cells treated with activation stimuli and the Lck inhibitor. The cells were stimulated with anti-IgM, CD40L, or, via Toll-like receptors (TLRs) using CpG oligonucleotides (ODN 2006 sequence).

Figure 3.6 shows that compared to the unstimulated control, all stimuli

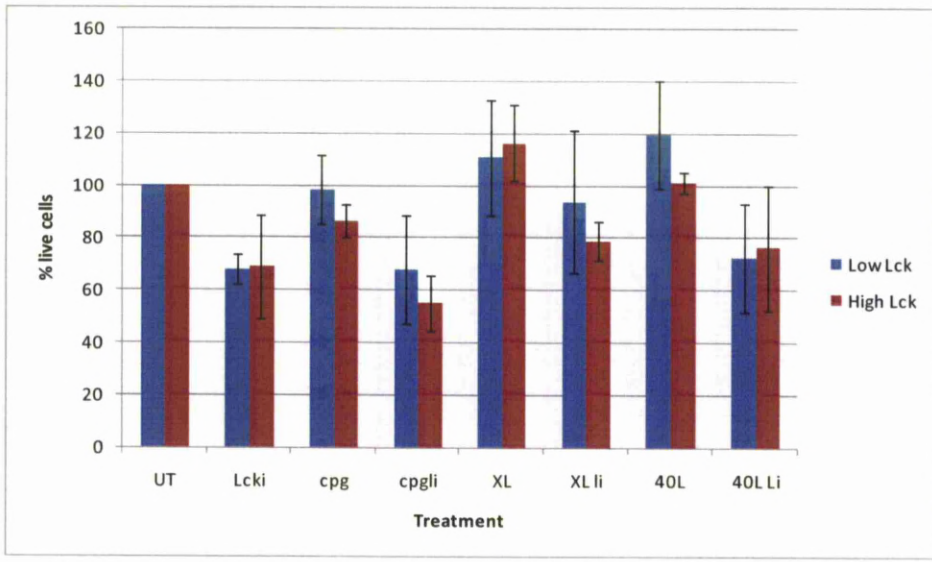


Figure 3.6 The level of Lck protein does not influence the induction of TLR, BCR or CD40L-induced survival. Cells from three high versus three low Lck expressing clones were stimulated for 48 hours with CpG at 0.5μg/ml, anti-IgM at 10μg/ml, CD40L at 200ng/ml coupled to enhancer at 1μg/ml in the presence or absence of 1μM Lck inhibitor. Cell survival was assessed using a $DiOC_6$/PI apoptosis assay and expressed as a percentage of untreated cell survival. Data represents the mean and error bars indicate the standard deviation of two experiments.

employed caused a different degree of preservation in cell viability. This apparent increase in cell viability reflects the protection of stimulated cells from spontaneous apoptosis that took place in unstimulated cells during the first 24 hours of cell culture. Furthermore, figure 3.6 also shows that the level of Lck expression did not seem to influence the protection given to CLL cells by the stimuli used. However, Lck inhibition either greatly reduced or prevented the pro-survival effects of BCR crosslinking ($p<0.1$), CD40 ligation ($p<0.05$) and TLR stimulation ($p<0.05$). This suggests that Lck activation is more important than expression in terms of providing cytoprotection to CLL cells, particularly with respect to the context of the pro-survival stimuli used in this study. The signalling pathways employed by BCR crosslinking, CD40 ligation and TLR stimulation to promote CLL cell survival are distinct from each other with respect to the different combinations of protein interactions and post-translational modifications within their respective signalling pathways. That Lck inhibition blocks the pro-survival effects of all these stimuli would suggest that the cytoprotective effect of Lck cannot be compensated by the presence of other pro-survival signals.

3.3.4 The mechanism of the prevention of apoptosis of CLL cells by Lck

The experiments described so far in this Chapter strongly suggest that Lck in both unstimulated and stimulated CLL cells plays a cytoprotective role.

The principal mechanism of cytoprotection in cells involves the maintenance of the balance of expression of proteins involved in the induction and prevention of apoptosis, with an emphasis on the expression of proteins involved in prevention. These proteins that are involved in the prevention of apoptosis belong to either anti-apoptotic Bcl-2 family proteins, or a family of proteins collectively known as inhibitors of apoptosis proteins (IAPs). Which of these proteins protects cells from apoptosis depends on upstream signals as well as on the circumstances in which the cells become prone to die and therefore require cytoprotection.

For the analysis of the anti-apoptotic effects of Lck I chose B-cell lymphoma extra long (Bcl-xL) and Mcl-1 as representatives of anti-apoptotic Bcl-2 family proteins, and cIAP1, cIAP2 and X-linked inhibitor of apoptosis protein (XIAP) as representatives of IAPs. It was assumed that changes in the expression of these proteins would be most informative, because all have been reported to be involved in cell cytoprotection of CLL cells [191-193].

3.3.4.1 CLL cell incubation with the Lck inhibitor causes conversion of Bcl-xL into a 17-18kDa proteolytic fragment

Figure 3.7 shows the changes in Bcl-xL expression in unstimulated CLL cells (A) and HCL cells (B) during 20 hours incubation with or without the Lck inhibitor. HCs were employed as a negative control since they did not

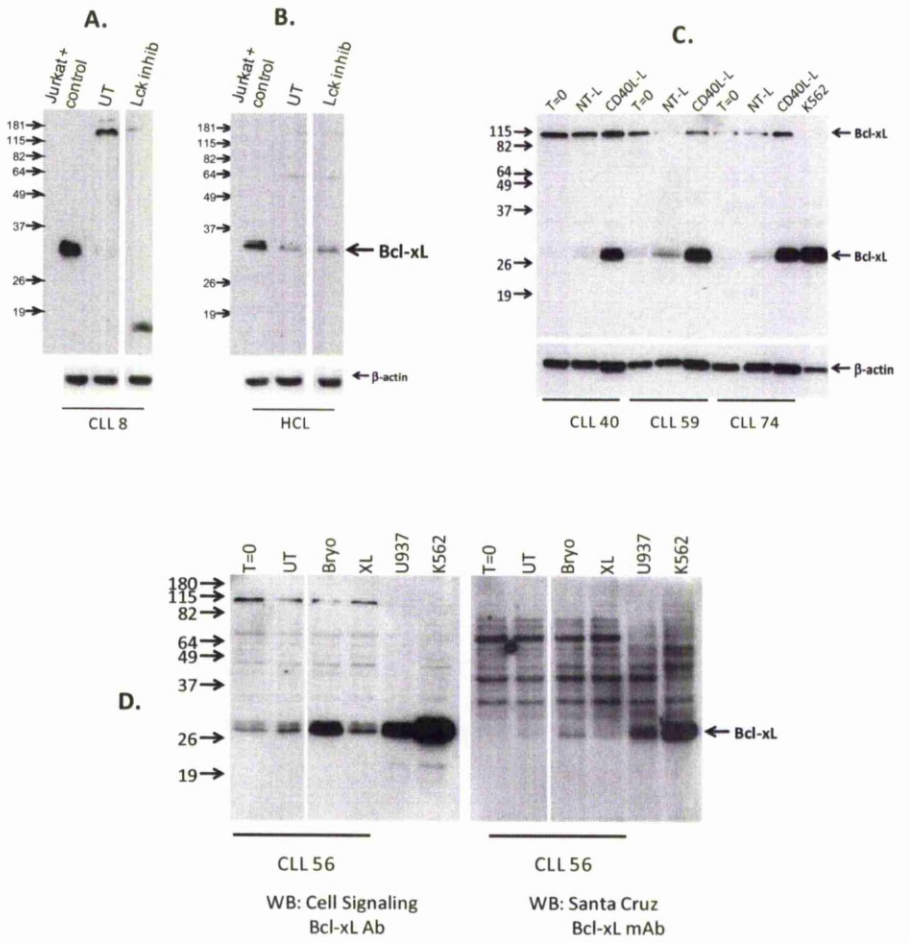


Figure 3.7. Inhibition of Lck causes Bcl-xL degradation in CLL cells. CLL cells (CLL8) (A) or HCs (B) were incubated for 20 hours in the presence or absence of the Lck inhibitor (1μM. The Bcl-xL protein was then visualised by Western blotting with a polyclonal anti-Bcl-xL antibody (Cell Signaling). (C) Three more CLL cases were either pelleted immediately post-thawing (T0) or cultured in the presence of untransfected fibroblasts (NT-L) or fibroblasts transfected with CD40 ligand (CD40L-L) for 48 hours. Equal quantities of the cell line K562 were loaded as a positive control. (D) One CLL case was pelleted immediatley post-thawing (T0) or cultured for 24 hours in the presence or absence of 50nM bryostatin of 10μg/ml F(ab')2 anti IgM. U937 and K562 cell lines were included as a positive control. The blot was then either probed with an anti-Bcl-xL anrtibody from Cell Signalling or one purchased from Santa Cruz. Results are representative of at least two experiments.

express Lck. The different molecular weight forms of Bcl-xL were visualised by Western blotting with a specific polyclonal antibody

A comparison of Bcl-xL expression in cell lysates of Jurkat T cells and CLL cells shows that the level of Bcl-xL at the expected molecular weight of 30kDa is considerably less in CLL cells than in Jurkat T cells (Figure 3.7A). Similar results were obtained when Bcl-xL expression in CLL cells was compared to that in a myeloid cell line, K562 (Figure 3.7C). Bcl-xL expression in CLL cells could be induced by culturing the cells on CD40L-expressing fibroblasts (Figure 3.7C). Interestingly, CLL cell lysates showed the presence of an extra band at 120kDa that was reactive with the Bcl-xL antibody we used (from Cell Signaling). This band was not present in either T cells or in K562 cells, and its presence in CLL cell lysates was not affected by culture of CLL cells on CD40L-expressing fibroblasts. Considering that Bcl-xL is known to spontaneously form dimers and tetramers [194, 195], the presence of the 120kDa band in CLL cell lysates suggests the presence of Bcl-xL tetramers, and that Bcl-xL expression in these cells is almost exclusively in this format.

I attempted to confirm that the 120kDa band was a tetrameric form of Bcl-xL by using other Bcl-xL-directed antibodies. Figure 3.7 shows a sample Western blot comparing Bcl-xL expression in CLL cell lysates using antibodies from two different manufacturers, Cell Signaling and Santa

Cruz. Both antibodies were able to detect the 30kDa Bcl-xL band in CLL and other cell lysates, and confirm that this band is likely to be Bcl-xL. However, only the antibody from Cell Signaling was able to detect the additional 120kDa band in CLL cell lysates. The antibody from Santa Cruz reacted with a variety of major bands corresponding to different molecular weights, and the pattern of reactivity was not consistent between the lysates derived from different cell types used on the Western blot. Thus, the identity of the 120kDa band could not be confirmed as a definite tetramer of Bcl-xL. Nevertheless, the fact that the Cell Signaling antibody primarily reacts with only two bands in Western blots of CLL cell lysates and only a single (30kDa) band in Western blots of other cell types indicates that Bcl-xL specificity can be assigned to the Cell Signaling antibody, and further suggests that the 120kDa band could be tetrameric Bcl-xL.

When I treated CLL cells with the Lck inhibitor, the pattern of antibody reactivity on Western blots of CLL cell lysates was completely altered (Figure 3.7A) The 120 kDa tetrameric as well as the 30kDa monomeric forms of Bcl-xL were almost completely absent and the only clear immunoreactive band was a new 17kDa protein previously identified as a pro-apoptotic proteolytic fragment of Bcl-xL [196].

In contrast to CLL cells, HCL cells contain primarily Bcl-xL monomer (Figure 3.7B). HCL cell lysates, like CLL cells, also contained the 120kDa

band associated with the Bcl-xL tetramer, however, an additional band corresponding in molecular weight to a 60kDa dimer of Bcl-xL was also present. However, these additional bands in the HCL cell lysates were weak in comparison to that of the Bcl-xL monomer. This suggests that if the 60 and 120kDa bands are Bcl-xL oligomers, the process of formation of these oligomers is different between CLL and HCL cells. That is, CLL cells favour the formation of tetrameric forms of Bcl-xL, whereas HCL cells favour the maintenance of the monomeric form. An additional difference between HCL and CLL cells was that incubation of HCL cells with the Lck inhibitor failed to induce expression of the 18kDa Bcl-xL degradation product. Since HCL cell viability is unaffected by the Lck inhibitor whereas CLL cell viability is reduced, the results of this experiment suggest that the appearance of the 18kDa Bcl-xL degradation product is likely associated with the induction of cell death. Finally, in the experiment represented by this Figure (Figure 3.7B) a reduced amount of Jurkat T-cell lysate was employed for control in order to avoid the excessive sample loading seen in Figure 3.7A.

To investigate further the mechanism of the conversion of Bcl-xL to the 18kDa fragment in the presence of the Lck inhibitor, CLL cells were additionally cultured with Z-Val-DL-Ap-fluoromethylketone (Z-vad). Z-vad inhibits protein degradation caused by caspases, and therefore could

allow identification of what causes the degradation of Bcl-xL in these cells.

3.3.4.2 Bcl-xL degradation caused by Lck inhibition is caspase-mediated.

Figure 3.8 shows an experiment in which CLL cells were incubated with the Lck inhibitor alone or in combination with the caspase inhibitor Z-vad.

As already seen in Figure 3.7A, inhibition of Lck resulted in the appearance of an 18kDa fragment of Bcl-xL and a reduction in the intensity of the 30kDa and 120kDa Bcl-xL bands. Co-incubation of CLL cells with Z-vad inhibited this effect of the Lck inhibitor. Both the formation of 18kDa fragment of Bcl-xL as well as the reduction in intensity of the 30kDa and 120kDa Bcl-xL bands caused by the presence of Lck inhibitor was completely blocked by Z-vad. This indicates that in the absence of Lck cytoprotection, apoptosis may be activated by a pro-apoptotic protein which can be neutralised by caspase inhibition. The most likely candidate for such a protein is the second mitochondrial activator of caspases/direct IAP binding protein with low pI (smac/DIABLO) which promotes apoptosis by binding to IAPs and preventing them from inhibiting caspases [197]. It is conceivable that some leakage of this protein from mitochondria occurs also in quiescent cells where it is neutralised and directed to proteasomal degradation by a sufficiently high

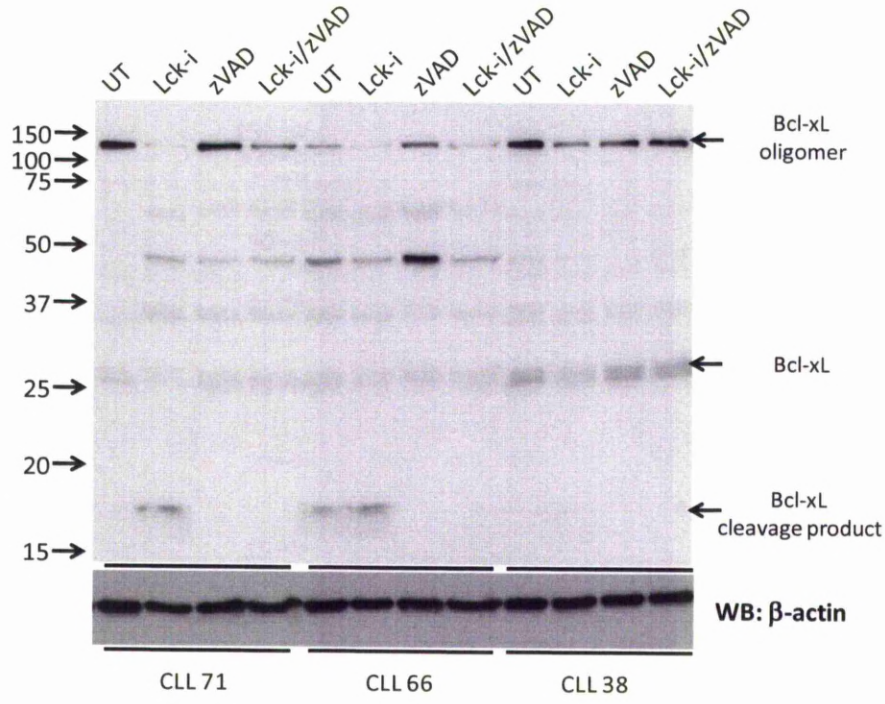


Figure 3.8 Degradation of Bcl-xL is caspase-dependent. The cells from 3 CLL cases were incubated for 48h in the presence or absence of either the Lck inhibitor (1μM) or Z-vad (500nM), or both. The figure shows Bcl-xL degradation (indicated by the appearance of 18kDa fragment and disappearance of the 30kDa and 120kDa Bcl-xL bands). This figure is representative of the same experiment performed on an additional 4 CLL cases with the same overall result.

concentration of IAPs [198]. Thus, Lck inhibition likely results in the lowering of the IAPs concentration to the level where they are fully neutralised by smac, allowing caspases to exert their apoptotic effect. This caspase involvement is supported by the absence of Bcl-xL degradation when samples treated with the Lck inhibitor also contained the caspase inhibitor Z-vad, confirming that this degradation is caused by caspases, most likely caspase 3, when IAPs are inhibited by smac. Thus the experiment in Figure 3.8 suggests that the contribution of Lck to CLL cell cytoprotection may be based on the participation of Lck in cell signalling pathways that stimulate synthesis of IAPs resulting in a change in the balance between pro-apoptotic smac and anti-apoptotic IAPs in favour of the latter.

3.3.4.3 Lck inhibition stimulates caspase activation and induces a reduction in XIAP, Mcl-1 and c-IAP2 expression in CLL cells.

In an attempt to provide experimental evidence for the proposal that the stimulation of IAP production may play a role in cytoprotection of CLL cells by Lck, I first examined the effect of Lck inhibition on the proteolytic cleavage of poly-(ADP-ribose) polymerase (PARP). PARP is a substrate of caspase 3, and proteolytic cleavage of PARP is seen as a marker of caspase 3 activation and induction of apoptosis [199, 200]. Figure 3.9A shows that incubation of CLL cells with the Lck inhibitor induced the appearance of the lower molecular weight band that is associated with caspase 3-

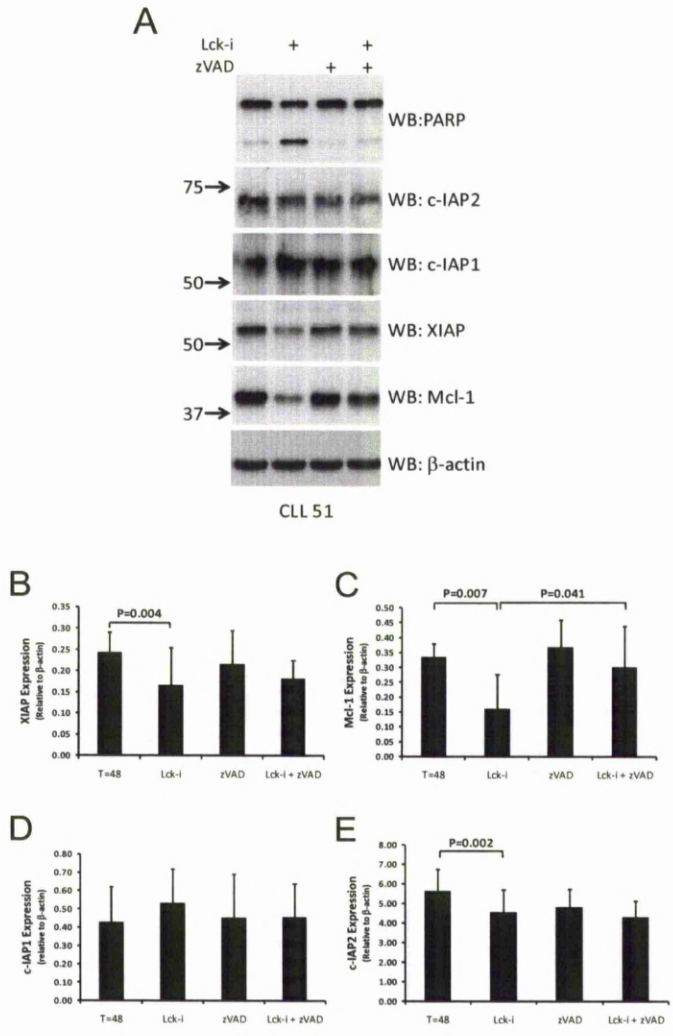


Figure 3.9 Lck inhibition induces PARP cleavage as well as reduction of XIAP, cIAP2 and Mcl-1 protein levels. CLL cells were treated for 48h in the presence or absence of either the Lck inhibitor (1μM) or Z-vad (500nM), or both. **A)** Western blots were probed for the presence of the indicated proteins. This figure is representative of the same experiment performed on 7 different CLL cases. **B – E)** Quantitative representation of expression for the indicated proteins in CLL cell lysates relative to the level of β-actin. n=7 for each antibody used. Statistical significance was established using a Student's t-test for paired data.

mediated cleavage of PARP. Furthermore, this figure also shows that the presence of Z-vad inhibited the formation of this band. These data suggest that Lck inhibition induces the activation of caspase 3 in CLL cells.

I next investigated the effect of Lck inhibition on IAP expression in CLL cells. Figure 3.9 shows that treatment of CLL cells with the Lck inhibitor led to small but significant reductions in expression of XIAP and cIAP2, but not cIAP1. Mcl-1 is a Bcl2 family protein whose expression in CLL and other cells is linked to apoptosis resistance. When I investigated the effects of Lck inhibition on the levels of this protein in CLL cells I found a similar reduction in expression (Figure 3.9A and C). The difference between Mcl1 and IAP proteins became apparent when I additionally treated the CLL cells with Z-vad. The presence of Z-vad in Lck-inhibited CLL cells reversed the reduction in Mcl1 expression (Figure 3.9C), but was without significant effect on the reduction of XIAP (Figure 3.9B) and cIAP2 (Figure 3.9E) expression. These results suggest that Lck may regulate the expression of Mcl1 and XIAP/cIAP2 through different mechanisms.

Taken together, the results of this section demonstrate that Lck inhibition in CLL cells results in the activation of caspases leading to the cleavage of proteins such as PARP and Mcl-1. Furthermore, these results also suggest that caspase activation in CLL cells may be caused by changes in Lck-

mediated induction of XIAP and cIAP2 expression. Thus, Lck inhibition leads to a reduction in XIAP and cIAP2 levels in CLL cells, and this results in CLL cells becoming more sensitive to the induction of apoptosis by smac/DIABLO.

3.3.4.4 Lck inhibition stimulates increased expression of Bim in CLL cells.

An important mediator of the intrinsic apoptosis pathway is the BH3-only protein Bim [201]. This member of the Bcl2 family of proteins binds to Bcl2 and allows the release of BAX and BAK, which are then able to promote permeabilization of mitochondrial membranes to result in the release of proteins such as smac/DIABLO and cause caspase activation and induction of apoptosis. Thus, controlling Bim expression may be important to the mechanism of cell cytoprotection provided by Lck.

Figure 3.10A shows that incubation of CLL cells for 48h led to a spontaneous increase in the levels of BimEL and BimL in two out of the three CLL cases shown. Treatment of CLL cells with the Lck inhibitor over this time frame induced a significant further increase in Bim, in particular BimEL, expression (Figure 3.10A and B). Taken together, these results suggest that Lck cytoprotection of CLL cells is at least partially regulated through the control of Bim expression.

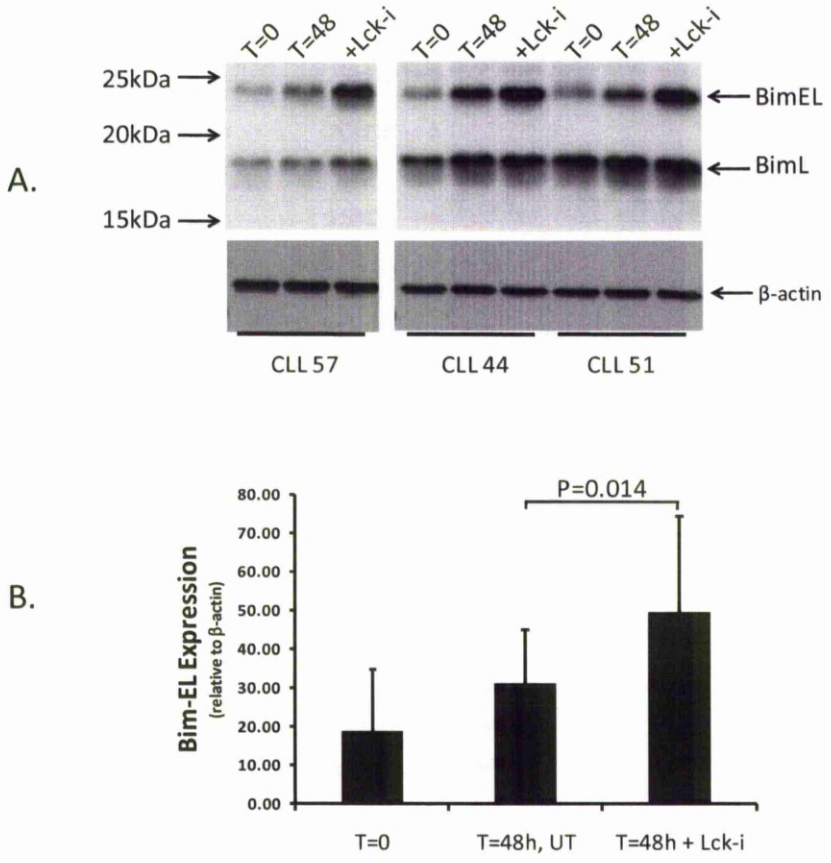


Figure 3.10 Lck inhibition induces an increase in BimEL expression in CLL cells. CLL cells were incubated for 48h in the presence and absence of Lck inhibitor (1μM). **A)** Western blot analysis of Bim expression in CLL cell lysates. This figure is representative of the same experiment performed on 4 additional CLL cell clones. **B)** Quantitative assessment of BimEL expression in CLL cells. Statistical significance was assessed using a Student's t-test for paired data (n=7).

3.3.4.5 The role of Lck in the activation of ERK by constitutive stimuli activated in CLL cells during prolonged *in vitro* culture

An important mechanism controlling the level of Bim expression in cells is the post-translational modification of this protein by ERK which results in its destruction by the cell proteosome. In this section I investigated the role of ERK because of the known role of this pathway in CLL cell cytoprotection, and because, in T cells, Lck is known to participate in signalling via this pathway [202-204].

It has been known for some time and now confirmed in figure 3.11 that *in vitro* unstimulated CLL cells contain phosphorylated ERK that varies in a case-dependent manner [205]. This was more recently followed by studies employing MEK inhibitors to demonstrate importance of this ERK phosphorylation/activation for CLL cytoprotection under various conditions [53, 206].

The present studies of the role of Lck in the activation of ERK were preceded by studies in our laboratory demonstrating that active ERK is differentially expressed in prognostically different subgroups of CLL, and that different cells respond differently to BCR stimulation as regards ERK phosphorylation and preservation of cell viability [161]. It was therefore of interest to see whether activation of ERK is responsible for the

improvement in cell viability of BCR stimulated cells and whether this activation of ERK depends on Lck.

The contribution of Lck to the activation of ERK in CLL cells was first explored in unstimulated cells cultured in the presence or absence of the Lck inhibitor for up to 24 hours. The results are shown in figure 3.11.

Figure 3.11 shows that immediately *ex vivo* (0hr), cells from different CLL cell cases contain different levels of phosphorylated ERK ranging from very strong (CLL12) to almost undetectable (CLL23). It was assumed that this activation of ERK is a consequence of *in vivo* cell stimulation. This assumption is supported by decrease of phosphorylated ERK in the first 2 hours of *in vitro* incubation when the cells are deprived of *in vivo* stimuli. The comparatively low and relatively constant level of ERK phosphorylation seen during subsequent 8 to 24 hours of culture may represent the background level of ERK activation required for cytoprotection of quiescent cells. The Lck inhibitor had little or no effect on this low-level phosphorylation suggesting that it is maintained by a process that does not require Lck activity. However, in cases CLL1, CLL12 and CLL8 ERK became again variably rephosphorylated during this period, and this rephosphorylation was inhibited by the presence of the Lck inhibitor.

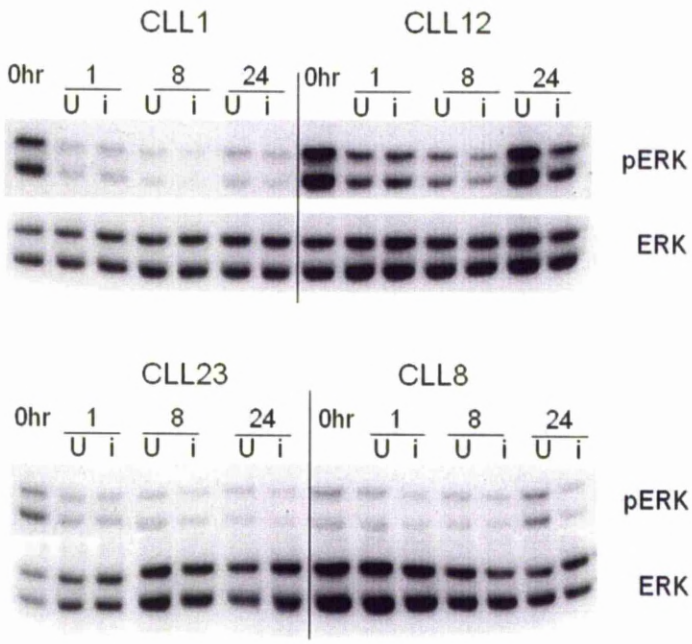


Figure 3.11 Reappearance of ERK phosphorylation in short term culture in some CLL cells is Lck-dependent In order to determine changes in ERK phosphorylation/activation in CLL cells during 24 hours culture, the stored frozen cells were thawed, washed and lysed to estimate the degree of ERK phosphorylation in cells tested immediately *ex vivo* (T0). The remaining cells were then suspended in the culture medium and left to recover for 1 hour at 37°C before addition of the Lck inhibitor (i) to 1 aliquot of the cell suspension leaving the other aliquot untreated (U) to serve as a control for each of the timepoints. The cell culture was then continued for 24 hours taking the samples of the treated and untreated cells at indicated times. Results are representative of three repeats.

This rephosphorylation of ERK was unexpected and raised the question whether initial dephosphorylation and subsequent rephosphorylation always take place under these culture conditions, whether rephosphorylation persists beyond 24 hour incubation and whether in all cases requires active Lck (as for CLL12).

For the estimation of changes in ERK phosphorylation in the presence or absence of the Lck inhibitor during an extended 72 hour culture, the cells were equally treated as described in the legend of figure 3.11. Figure 3.12 shows that the degree of ERK phosphorylation at time 0 varied from case to case. In three of the cases shown in figure 3.12A this is followed by rapid dephosphorylation during the first hour of incubation, regardless of whether or not the samples contained the Lck inhibitor. In these three cases ERK became rephosphorylated again between 8 and 24 hours as already seen in figure 3.11. From then on in untreated cells ERK remained phosphorylated until the end of the 72 hour incubation period, but in the inhibitor treated cells this phosphorylation was strongly inhibited. In contrast, in the three cases shown in figure 3.12B, ERK did not become initially dephosphorylated, the phosphorylation was maintained throughout the incubation at a relatively constant level. This level of activation was either not affected by the Lck inhibitor or slightly decreased in some samples at a very early stage of incubation (up to 8 hours).

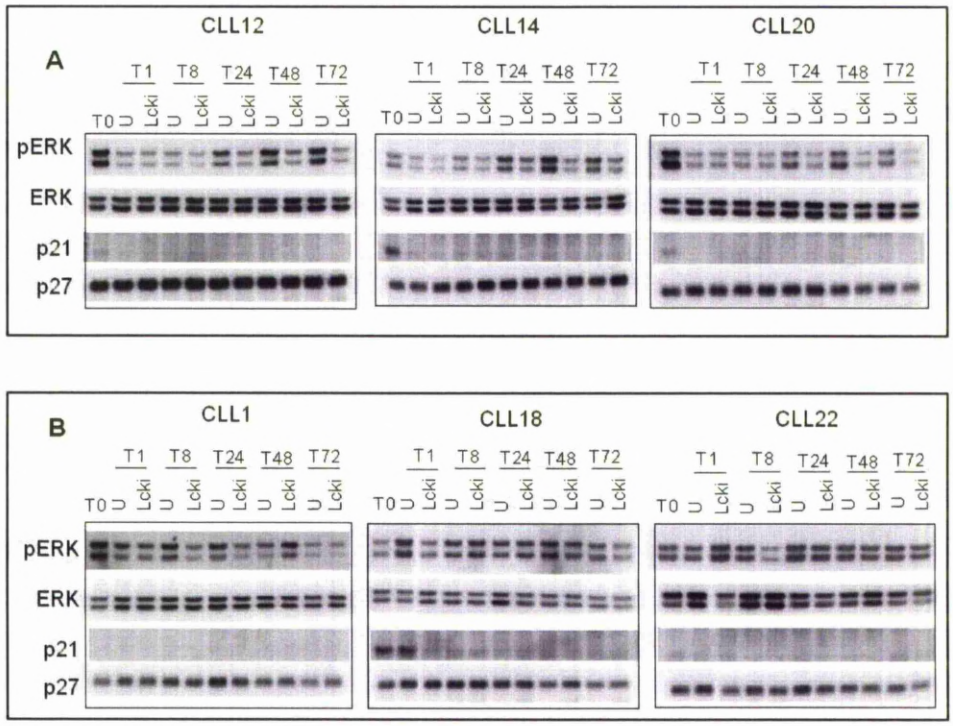


Figure 3.12 The Lck inhibitor decreases the reappearance of ERK phosphorylation in some CLL cases. Western blots showing the changes in ERK phosphorylation and expression of p21 and p27 in 6 different CLL clones cultured in the presence or absence of the Lck inhibitor for up to 72 hours. Results are representative of three experiments.

These results suggest that CLL cases can be split into 2 groups. In the first group the rephosphorylation of ERK at 24 hours is dependent on the activity of Lck (Figures 3.11 and 3.12A), whereas Lck does not contribute to the rephosphorylation of ERK in the second group (Figure 3.12B). To investigate the mechanism of ERK phosphorylation in this second group of CLL cases, I used the general SFK inhibitor PP1. Figure 3.13 shows that the presence of PP1 inhibited the reappearance of phosphorylated ERK in all cases. This suggests that a Src kinase other than Lck is responsible for rephosphorylation of ERK in the malignant cells from this second group of CLL.

3.3.4.6 The role of Lck expression in determining CLL cell survival in prolonged *in vitro* culture

The data in the above sections suggest that Lck may be important for CLL cell cytoprotection through a mechanism involving activation of ERK and maintenance of low levels of Bim. If this model is correct then it is likely to depend on either Lck activity or expression levels, or a combination of both.

In Chapter 2 I show that CLL cells from different cases can express widely ranging levels of Lck. To test whether the level of Lck expression plays a role in CLL cell survival, I incubated CLL cells for 72, 96 and 120 hours

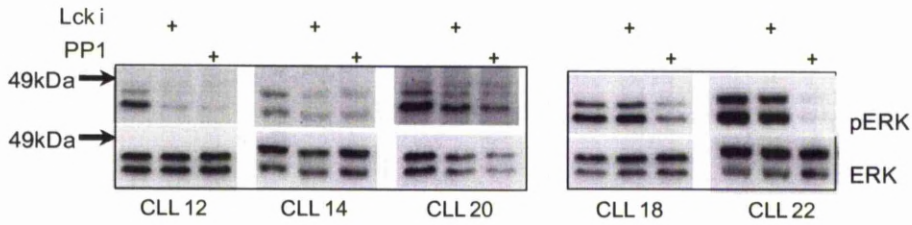


Figure 3.13. Comparison of the general Src family kinase inhibitor PP1 and the Lck inhibitor on rephosphorylation of ERK in CLL cells. Western blots showing the changes in ERK phosphorylation in CLL cells incubated with the Lck inhibitor (1μM) or with the general Src family kinase inhibitor PP1 (10μM) for 24 hours. Results are representative of four repeats.

and measured the percentage of viable cells remaining in these cultures. Figure 3.14 shows that the percentage of viable cells remaining in culture after these incubation times was significantly lower for CLL cells expressing low levels of Lck than for those cells expressing high levels of Lck. These results suggest that Lck protein levels are an important factor for Lck-mediated cytoprotection of CLL cells.

I next examined whether Lck protein expression was important for ERK rephosphorylation and Bim expression in CLL cells that had been cultured for 72h. Figure 3.15 shows a Western blot analysis of Bim and pERK expression in CLL cells from clones bearing low levels of Lck (Figure 3.15A) and from clones bearing high levels of Lck (Figure 3.15B). BimEL expression was upregulated in 4 of the 6 low Lck expressing CLL cases I analysed. However, the increase in Bim expression was not always associated with low levels of pERK. In the high Lck expressing CLL cell clones, Bim expression showed upregulation in 5 of the 8 cases analysed, and, like with low Lck expressing CLL cell clones, the increase in Bim expression did not always correlate with low levels of pERK. The only difference between high and low Lck expressing CLL clones seemed to be the level of pERK. The density of the pERK bands seemed to be higher in CLL cases bearing high levels of Lck protein. Thus, there appears to be no relationship between ERK rephosphorylation and expression of Bim in

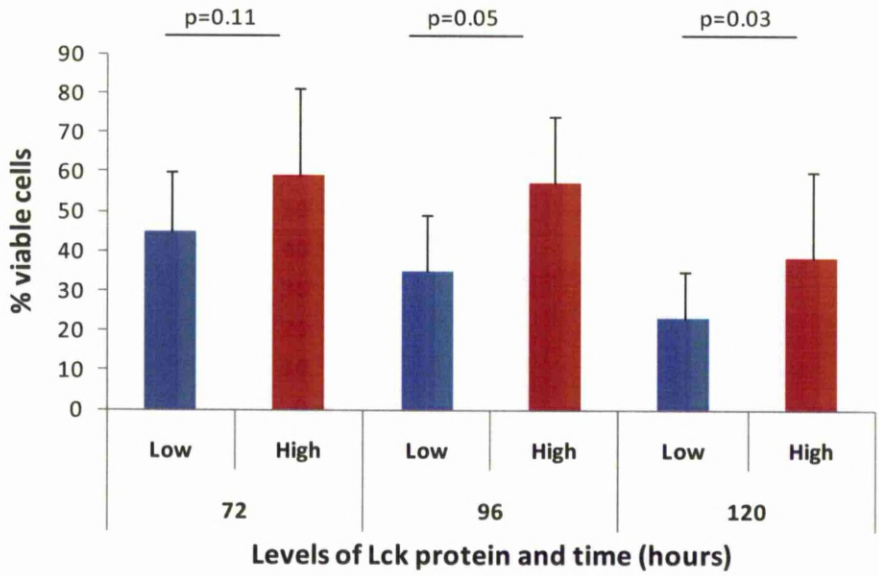


Figure 3.14 Levels of Lck correlate with spontaneous apoptosis of CLL clones. CLL cells from five low (■) and five high (■) Lck expressing clones were seeded at $4x10^6$ cells/ml and cultured for 72, 96 or 120 hours prior to the measurement of viability. CLL cell viability was assessed by FACS analysis of the percentage of $DiOC_6$ positive / PI negative cells (live healthy cells). The data is presented as mean±SD of 2 experiments. Statistical analysis was analysed using a Mann-Whitney U test where $p<0.05$ is considered statistically significant.

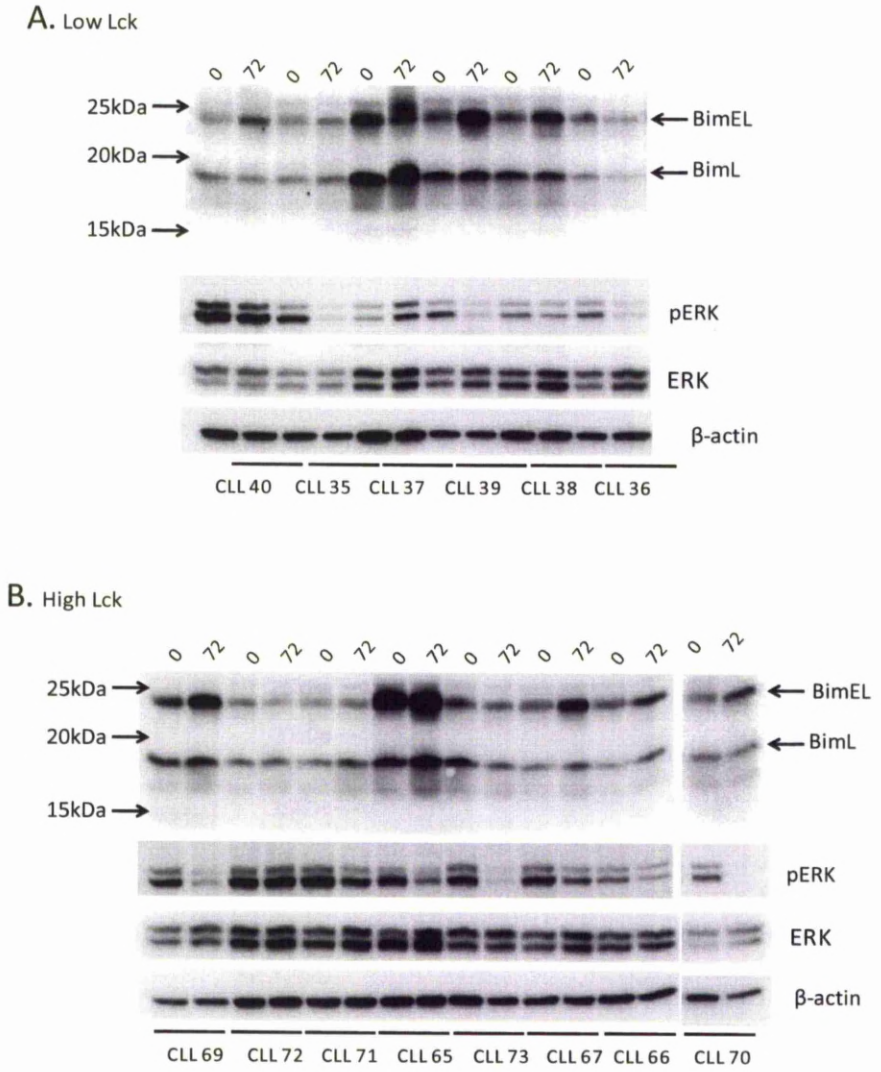


Figure 3.15 Bim levels at 0 and 72 hours of culture in low versus high Lck expressing cases. 14 CLL cases were thawed and resuspended at $4x10^6$ cells/ml. 1ml was either pelleted immediately (0) or cultured unstimulated for 72 hours (72). Cells were pelleted, protein determination carried out and 10μg total protein subjected to Western blotting for Bim, phosphorylated and total ERK or β-actin as a loading control. Results are representative of two experiments.

CLL cells. Taken together with the survival data, these results suggest that the survival advantage given to high Lck expressing CLL clones may be due to higher levels of constitutive ERK activation rather than control of Bim expression. Finally, because Lck inhibition stimulates Bim expression in CLL cells (Figure 3.10), the results presented here implicate additional mechanisms, perhaps involving other signalling pathways, in the cytoprotection provided by Lck to CLL cells.

3.4 Discussion

The work in this chapter was the first to assign some specific functional role of Lck in CLL cells. In the study which first demonstrated Lck expression in CLL cells [4], it was suggested that this expression indicates the B-1 cell origin of CLL cells, and that in B-1 cells, Lck plays a role in the self renewal of these cells. However the nature of the mechanism by which this enzyme could contribute to this self renewal and whether Lck has a parallel role in self renewal of CLL cells has not been established. Therefore in order to determine whether Lck has some specific non-redundant functional role in CLL cells, siRNA knockdown of Lck mRNA was attempted to inhibit Lck protein production in these cells.

siRNA was already successfully used in our laboratory to suppress production of some proteins, such a c-Abl, which rapidly turn over in quiescent cells [177] and this was repeated in this study. However this

technique did not result in the significant down-regulation of Lck during the cell culture period when these non-proliferating cells are still viable. It was therefore decided to use a specific inhibitor to explore Lck function(s) which are not shared by other SFKs in quiescent as well as stimulated CLL cells.

In the current study a recently developed compound, 4-Amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2-d]pyrimidin-7-yl-cyclopentane, has been employed because it is supposed to have higher specificity for Lck than other previously used inhibitors [188]. Nevertheless, it was thought to be necessary to carry out an experiments to demonstrate the specificity of this inhibitor for Lck relative to that for Lyn (figure 3.2), because the latter enzyme is highly expressed in CLL cells where it has many functions equivalent to the functions of Lck in T cells. In addition, HCs which do not express Lck and whose viability is affected by the general SFK inhibitor PP1, served as a useful negative control for the specificity of the Lck inhibitor. As expected, this inhibitor had no effect on functional responses of HCs (figures 3.2) although these responses were strongly affected by PP1, a general inhibitor of SFKs (previous unpublished work from this department and [189]).

The Lck inhibitor was first employed at a range of concentrations to explore the potential role of Lck in *in vitro* survival of CLL cells and HCs.

In all CLL cells, but not in HCs, a different degree of decrease in viability was seen after 48 hours incubation with increasing concentrations of the inhibitor, generally reaching 50% change in viability at concentrations of about 1μM. When 56 different clones of CLL cells with either mutated or unmutated IgVH genes were then incubated with 1μM of this inhibitor and cell viability measured at 48 hours incubation, the unmutated cells showed a significantly larger decrease in viability than the mutated cells, while the viability of HCL controls again remained unchanged.

Previous work has shown that unmutated cells contain phosphorylated ERK and that this phosphorylation further increases in response to BCR stimulation which also prolongs *in vitro* survival of these cells. In contrast, mutated cells show strongly reduced response to BCR stimulation which in addition has little effect on the survival of these cells [161]. Since the activity of Lck in unmutated quiescent CLL cells was found to be several fold higher than in mutated cells [190], it seemed possible that the differences in the effect of Lck inhibitor on mutated and unmutated cells reflect the involvement of active Lck in a cytoprotective pathway activated through BCR stimulation that may involve ERK.

To examine whether Lck is involved in cytoprotection provided by stimulation of CLL cells via a number of other receptors besides BCR, the cells were stimulated with anti-IgM, CD40L and CpGs. These stimuli

protected cell viability to a variable degree. The ability of these stimuli to provide pro-survival signals was not dependent on Lck levels, but on Lck activity because the presence of the Lck inhibitor either completely or almost completely abolished the pro-survival effects of these stimuli. Thus, Lck activity is important with respect to its provision of cytoprotective effects to CLL cells. This notion is supported by previous work from this Department showing that the activity level of Lck in unmutated quiescent CLL cells is several fold higher than in mutated cells [190], and by the data presented in this thesis showing that unmutated CLL cells are more susceptible to Lck inhibition than are mutated CLL cells.

The relevance of these observations for the well established relationship between the presence or absence of IgVH gene mutation and disease prognosis is currently unclear. The present *in vitro* study only indicates that Lck is likely to be involved in cytoprotection of the cells *in vivo*. However since the effect of Lck on CLL cells involves ERK activation, Lck may also participate in cell proliferation that clearly takes place in the *in vivo* microenvironments which so far have not been successfully reproduced *in vitro*.

Recent studies of the *in vivo* CLL-cell turnover [207] suggest that the proliferative component (cell birth rate) is higher in active, progressive disease, which is generally observed in cases with unmutated IgVH genes

and unfavourable disease prognosis. This study indicates that in this group of patients, not only proliferation of the malignant cells, but also their survival might be particularly dependent on Lck-dependent signals triggered by stimulation of BCR and TLRs by *in vivo* antigens.

B cell survival depends on the activity of several pathways, the principal ones being ERK, Akt, NFκB and NF-AT. Each of these pathways might be differentially activated depending on the stage of cell maturation and the conditions under which the cells are protected. This raised a question of whether, as suggested previously [190], this cytoprotection involves ERK. Therefore, it was first examined whether CLL cells during prolonged *in vitro* culture contain phosphorylated/ activated ERK and whether this ERK phosphorylation in any way depends on active Lck. The results of the experiments designed to answer these questions showed that all CLL cells when examined directly *ex vivo* contain variable amounts of phosphorylated ERK which in most cases is not seen after 2 hours of cell culture where the cells are deprived of external stimuli. However in most cases phosphorylated ERK reappeared in the cells, usually between 8 and 24 hours of culture and, with regards to the role of Lck, this ERK reactivation showed two distinct patterns. In some cells the ERK rephosphorylation was inhibited by the Lck inhibitor, but in other cases this inhibitor had no effect. Surprisingly, inhibition of ERK phosphorylation using the MEK inhibitor had little or no effect on cell

viability measured at 48 hours, whereas the samples containing the Lck inhibitor always had a smaller number of viable cells than the untreated control, regardless of whether or not the Lck inhibitor affected ERK phosphorylation. Therefore it is possible that the principal cytoprotective effect of Lck either does not involve ERK activation, or that this activation proceeds by a MEK-independent pathway. Alternatively, Lck may rescue cells from apoptosis through Akt or NFκB-dependent mechanisms, while activation of ERK might serve another function such as decreasing the sensitivity of the cells to the level most appropriate for interaction with self antigens and thus protecting the cells from negative selection by these antigens [30].

In this chapter, the mechanism of cytoprotection of CLL cells by active Lck has been studied by analysis of Bcl-xL degradation following prolonged CLL cell incubation with the Lck inhibitor. The amount of Bcl-xL is generally decreased during apoptosis in parallel with the caspase 3-dependent generation of a 17-18kDa pro-apoptotic ΔN Bcl-xL fragment. During the present study this fragment was seen in unstimulated cells in the presence of the Lck inhibitor. This suggested that Lck protects CLL cells from apoptosis by a mechanism leading to the inhibition of an activator of caspases. The principal candidate protein with these properties is smac/DIABLO which binds IAPs and prevents them from inhibiting

caspases. This then further suggests that Lck may be involved in synthesis of IAPs that bind smac/DIABLO and direct it to proteasomal degradation.

Chapter 4

Role of Lck in BCR signalling in CLL cells

4.1. Introduction

The work in Chapter 3 showed that Lck functions in a cytoprotective role in CLL cells by modulating the balance between proteins involved in the induction and prevention of apoptosis. However, this might not be the only function for this SFK in these cells. Importantly, the major function of Lck in T cells is the regulation of TCR signalling where it phosphorylates ZAP70, among other proteins, to stimulate activation of downstream molecules. ZAP70 expression is also observed in the malignant cells from a subset of CLL cases. Here, the role of ZAP70 has been shown to potentiate BCR signalling in CLL cells. Since ZAP70 is a substrate of Lck in T cells, it seemed logical that it should also be a substrate of Lck in CLL cells. Thus, Lck may have a role to play in BCR signalling in CLL cells. The aim of this Chapter was to investigate this possibility.

4.2. Materials and methods

4.2.1 Cell isolation and culture

CLL samples were obtained and cultured as described in methods section 2.2.1

4.2.2 CD5 crosslinking

CD5 antibodies (BD Biosciences clone L17F12 or Santa Cruz Biotechnology clone UCH-T2) were washed in PBS to remove the 0.1% sodium azide included as a preservative as this would be cytotoxic and therefore affect the survival of CLL cells independent of any CD5 crosslinking specific affect. Antibodies were spun through an Ultracell YM-50 filter system (Millipore) at 14,000rpm, the sodium azide buffer was discarded and the antibody (which remains in the filter column) was reuspended in PBS. Antibodies were either coated onto 24 well tissue culture plates for 12 hours at 4°C, washed and the cells then added to these wells or incubated directly with the cells at a final concentration of 5μg/ml. Survival of the CLL cells after 48 hours of incubation was assessed using the $DiOC_6$/PI apoptosis assay detailed in methods section 2.2.7.

4.2.3. Inhibitors and stimuli

All inhibitors were dissolved in DMSO, aliquoted and stored at -20°C. The The Lck inhibitor (4-Amino-5-(4-phenoxyphenyl)-7H-pyrrolo[3,2-

d]pyrimidin-7-yl-cyclopentane) was used at 1μM, U0126 was used at 10μM, lactacystin at 5μM, PD98059 at 10μM, RO-32-432 at 10μM and bisindolylmaleimide I at 1μM or 10μM, and all were purchased from Calbiochem. The caspase inhibitor (Z-Val-DL-Ap-fluoromethylketone) used at 500nM was purchased from Bachem (St Helens, UK).

$F(ab')_2$ fragments of goat anti–human IgM (Jackson ImmunoResearch Laboratories. were used at a final concentration of 10μg/ml for crosslinking/stimulating the BCR. CpG stimulation utilised 5μg/ml ODN2006 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3') oligonucleotides for human Toll-like receptor 9 (TLR9) stimulation (InvivoGen; Autogen Bioclear, Calne, UK).

4.2.3 Western blotting

As described in 2.2.3. In addition, blots were blocked in 5% milk dissolved in TBST for analysing the expression of proteins, or 5% BSA in TBST for analysis of phosphorylated proteins. Antibodies were prepared in TBST containing either 5% milk powder or 5% BSA as for blocking of blots.

The following primary antibodies were used (all at 1:1000): anti-Lck mouse monoclonal, anti-pERK mouse monoclonal, anti ERK rabbit polyclonal, anti pY551-Btk rabbit polyclonal and rabbit anti-Mcl-1 polyclonal (Santa Cruz Biotechnology). Anti-pSer473Akt mouse

monoclonal, anti-pIKK rabbit polyclonal, anti-pY319 ZAP70 rabbit polyclonal, anti-pY525-Syk rabbit polyclonal and anti-pY323-Syk rabbit polyclonal antibodies were purchased from Cell Signaling Technology. The total ZAP70 antibody was purchased from Signal Transduction laboratories. The following secondary antibodies were used: Goat anti-rabbit horseradish peroxidise (HRP) conjugated antibody, goat anti-mouse HRP-conjugated antibody (Santa Cruz Biotechnology). Tyrosine phosphorylation was assessed in immunoprecipitates using an anti-phosphotyrosine antibody (PY20) purchased from ICN Biomedicals (Ohio, U.S.A).

4.2.4. Immunoprecipitation

As detailed in methods section 3.2.5. The CD5 immunoprecipitating antibody (cloneUCH-T2) and the Lck immunoprecipitating antibody (mouse monocolonal, clone 3A5) were purchased from Santa Cruz Biotechnology. Immunoprecipitation buffers used are detailed in the appendix.

4.2.5. FACS analysis of CD25 levels

CD25 levels in response to CD5 crosslinking were assessed by FACS analysis as detailed in methods section 2.2.6 using an antibody from BD Biosciences.

4.3. Results

4.3.1 Examination of a potential role for Lck in the downregulation of BCR-induced signalling through association with CD5

The current paradigm explaining BCR signal transduction in CLL cells involves the activation of the SFK Lyn and, further downstream, of the tyrosine kinase Syk [208]. These signals are then further processed by CLL cells to transduce pro-survival and anti-apoptotic responses. A similar role for Lck could also be proposed within the BCR signaling cascade because of the known role of this SFK in the TCR signaling cascade. However, such a role for Lck in B cells has not been established. Rather, because Lck expression in B cell lineages is normally restricted to B-1 cells [4], and because B-1 cells also express CD5 [209] which is a known substrate of Lck in T cells [210] that serves as a scaffold for the protein tyrosine phosphatase SHP-1 [123], it is thought that Lck is responsible for the downregulation of BCR signaling. Support for this role of Lck in B-1 cells is provided by studies that show CD5 expression is linked to negative regulation of BCR signals in B-1 cells [162], and that show Lck expression in B-1 cells is necessary for this hyporesponsiveness to BCR engagement [109]. Since CLL cells express both CD5 and Lck, it seemed logical to assume that a function of Lck in these cells may be in conjunction with CD5. This possibility was therefore next examined.

Previous work by others has shown that CD5 crosslinking induces apoptosis of CLL cells [211, 212]. I attempted to repeat these experiments. However, I found that CLL cell viability was unaffected by the presence of CD5 antibodies (Figure 4.1A). Similar results were generated in experiments using either different antibodies, or different concentrations of the same antibody. Furthermore, I found that CD5 crosslinking had no effect on the viability of CLL cells matching the phenotypic criteria of those used in a previous study showing the pro-apoptotic effect of this stimulus.

I next tested whether I could show that CD5 antibodies induced any type of response in CLL cells. It has been described that crosslinking CD5 induces upregulation of the IL2 receptor (CD25) expression on T cells [213] and B cells [214]. When I examined CD25 expression on CLL cells that had been exposed to CD5 antibodies, I found that the expression of this protein had been upregulated (Figure 4.1B). This result demonstrates that CLL cells do respond to CD5 crosslinking, but that apoptosis, at least in my experiments, was not the result.

To investigate the role of Lck in the CD5 generated signals that resulted in increased expression of CD25 on CLL cells I used the Lck inhibitor. Figure 4.1B shows that CLL cells treated with the Lck inhibitor failed to upregulate CD25 in response to CD5 crosslinking. Thus, Lck in CLL cells

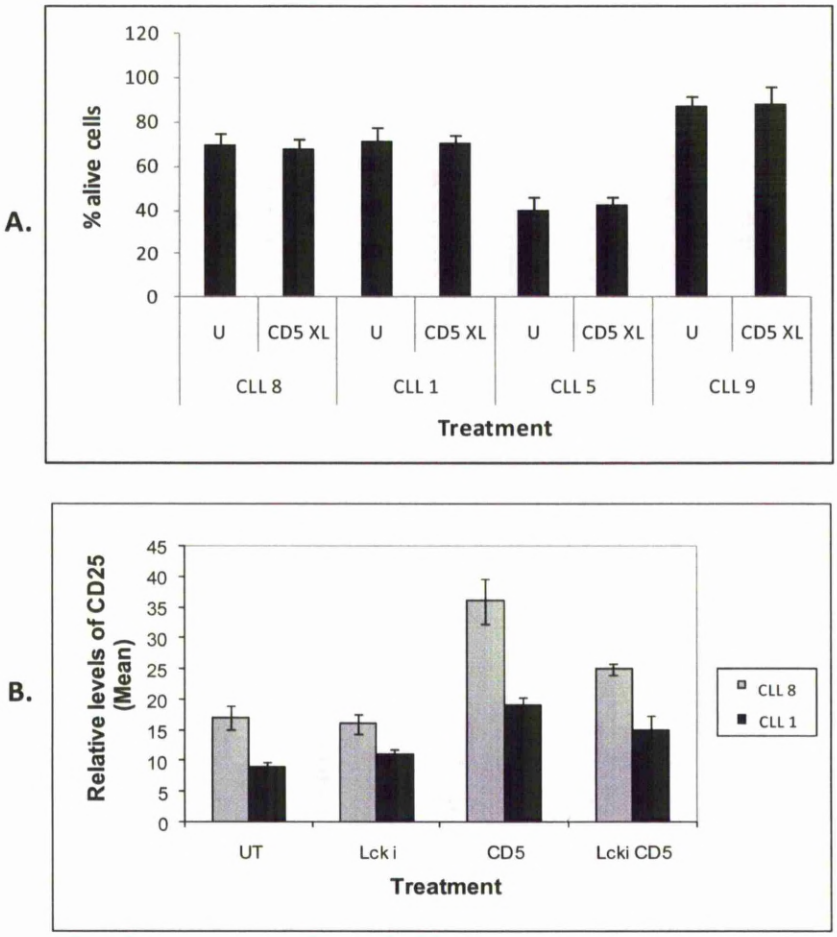


Figure 4.1 CD5 crosslinking did not induce CLL cell apoptosis but can upregulate CD25 in an Lck-dependent manner. Four CLL cases were treated with 50μg/ml anti-CD5 antibody for 48 hours and cell survival assesed using a $DiOC_6$/PI apoptosis assay (**A**) expressed as the mean and standard deviation of three experiments. Two CLL cases were treated as for A and CD25 expression was quantified by FACS analysis (**B**). Results are representative of two experiments.

may associate with CD5 and help to transduce signals from this protein.

In T cells Lck functions to phosphorylate CD5, and this process is then responsible for attracting SHP-1 to CD5 where it acts to downregulate TCR signals. A similar role for Lck in CLL cells has been suggested by Majolini [4]. To investigate CD5 phosphorylation in CLL cells I immunoprecipitated this protein and then probed Western blots of the immunoprecipitate with antibodies recognising phospho-tyrosine. Figure 4.2 shows that tyrosine phosphorylation of CD5 in Jurkat cells increases in cells cultured in the presence of the phosphotyrosine phosphatase inhibitor mpV(pic). This increase was most convincingly demonstrated in experiments using TX-100 lysis buffer, but was also observed in experiments using other buffers. However, I was unable to conclusively show that CD5 was tyrosine phosphorylated in CLL cells. The bands present in the CLL immunoprecipitates either migrated to a different position on the gel, or were too faint to allow analysis despite using $1x10^7$ cells. Thus, the role of Lck in CD5 phosphorylation could not be investigated in CLL cells in this study.

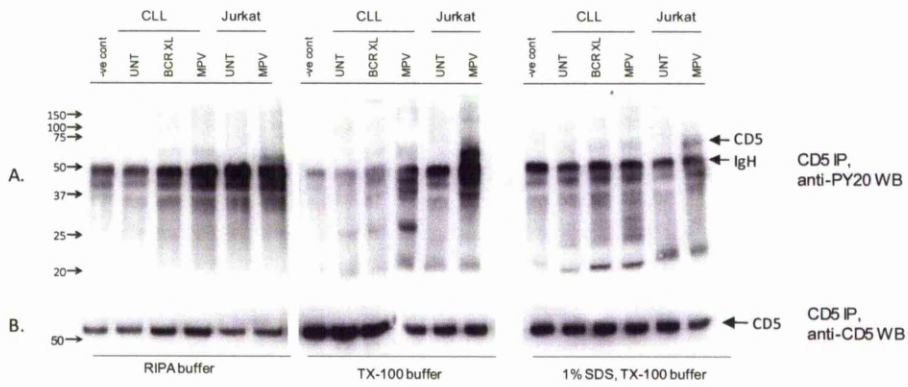


Figure 4.2 CD5 phosphorylation can be induced in the Jurkat cell line, but not in CLL cells, by MPV stimulation. Immunoprecipitation of CD5 from the Jurkat cell line or one CLL clone which expressed high levels of CD5 protein and then analysis of its tyrosine phosphorylation status (**A**) or validation that CD5 had been immunoprecipitated (**B**) was carried out using three different immunoprecipitation buffers (RIPA/ TX-100 or a TX-100 buffer containing 1% SDS). CLL cells were treated for 30 minutes with 10μg/ml F(ab')$_2$ anti-IgM and 100nM mpV(pic) (MPV) was used in both cell types as a positive control to induce CD5 phosphophorylation. Results are representative of two experiments.

4.3.2 Examination of a potential positive role for Lck in BCR-induced signalling in CLL cells

4.3.2.1 Role of Lck in BCR-mediated activation of MAPK, PI3K and NFκB signalling

The work describing a role for Lck in the modulation of BCR signals in B-1 cells is controversial. One study comparing BCR signals between peritoneal and splenic B-1 cells suggests that the hyporesponsiveness of peritoneal B-1 cells to BCR engagement is due to Lck expression [109]. However, another study has suggested that Lck expression enhances BCR signals in B-1 cells [126], while a third suggests that Lck has no role to play at all [127]. From these studies it is difficult to hypothesise a possible role for Lck in this context. However, data from Chapter 3 show that the pro-survival effects of BCR crosslinking can be blocked by treating CLL cells with the Lck inhibitor (Figure 3.4). Moreover, these data also show that BCR crosslinking induces changes to the activation of Lck (Figure 3.2). Considering that ZAP70 is expressed in the malignant cells of a subgroup of CLL cases, that ZAP70 is a predominant Lck substrate in T cells undergoing antigen receptor signalling, and that the function of ZAP70 in CLL cells is to enhance BCR signals, it seemed reasonable that Lck may function in CLL cells to enhance antigen receptor signals.

BCR crosslinking of CLL cells induces activation of several pathways including the MAPK, PI3K, and NFκB pathways. Activation of these particular pathways is characterised by the phosphorylation of the key kinases ERK, Akt and IKK, respectively. To assess the impact of Lck activity on BCR-induced signals in CLL cells, I used the Lck inhibitor and investigated its effects on BCR-mediated activation of ERK, Akt and IKK.

I first investigated the effects of the Lck inhibitor at different concentrations. Figure 4.3 shows the effect of increasing concentrations of Lck inhibitor on BCR-induced ERK, Akt and IKK phosphorylation. From this experiment, it seemed that the induction of IKK phosphorylation was particularly sensitive to Lck inhibition, whereas the induction of Akt and ERK phosphorylation required higher concentrations of Lck inhibitor before any effect could be seen. Because 1μM Lck inhibitor was the minimum concentration that produced maximum inhibition of BCR-induced ERK, Akt and IKK phosphorylation, this concentration was used for the rest of this study.

I next compared the effect of the Lck inhibitor on BCR and TLR signalling. Figure 4.4 shows that the presence of 1μM Lck inhibitor strongly reduced the increase in pERK levels that were stimulated by BCR crosslinking of CLL cells, and completely blocked BCR-induced

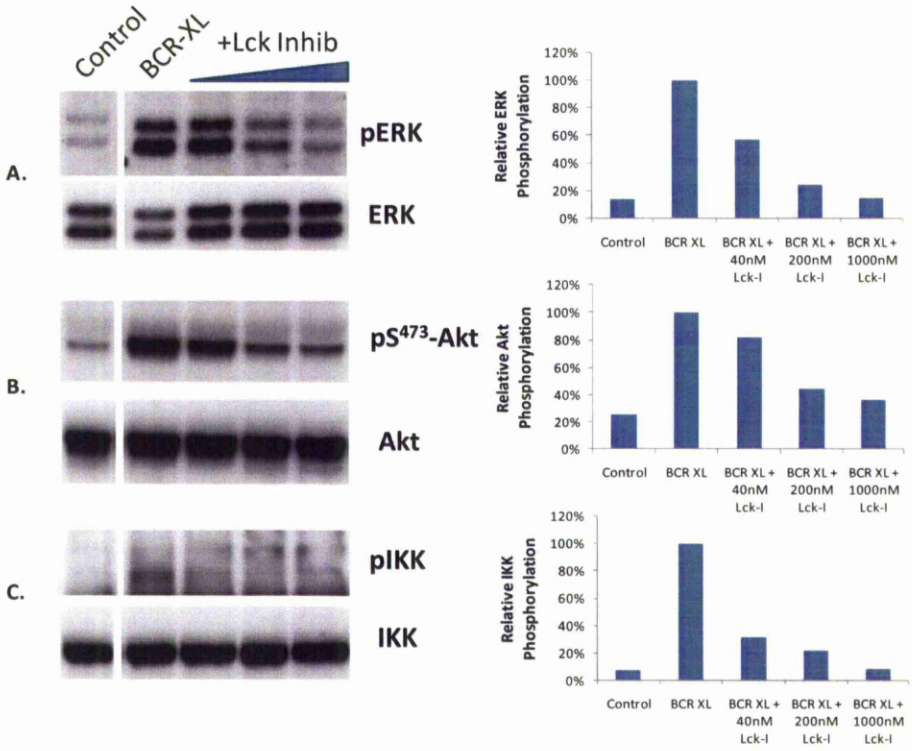


Figure 4.3 BCR crosslinking-induced ERK, Akt and IKK phosphorylation are inhibited by the Lck inhibitor. A dosage response of the effect of the Lck inhibitor on phosphorylation at 40nM, 200nM or 1000nM (1μM as used for the rest of this chapter). Densitometry was performed and is represented in the bar charts. Results are representative of three experiments.

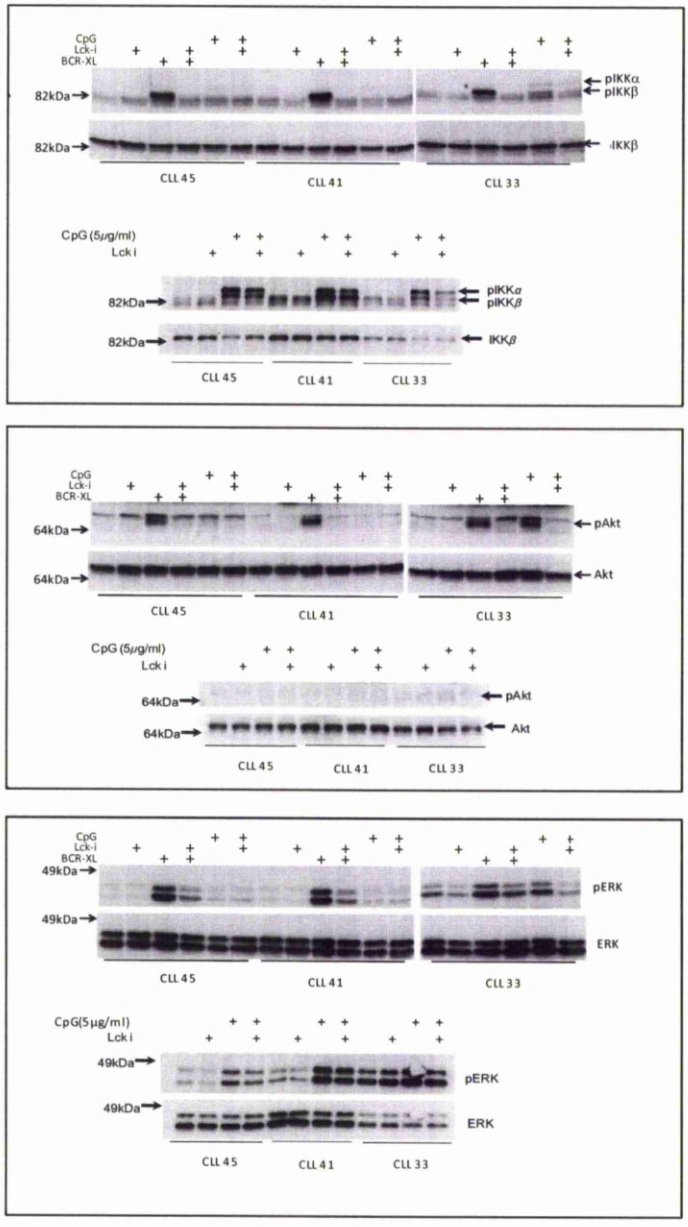


Figure 4.4.A. Phosphorylation of IKK, Akt or ERK by BCR crosslinking, 0.5μg/ml or 5μg/ml CpG stimulation in three CLL cases. Results are representative of three experiments.

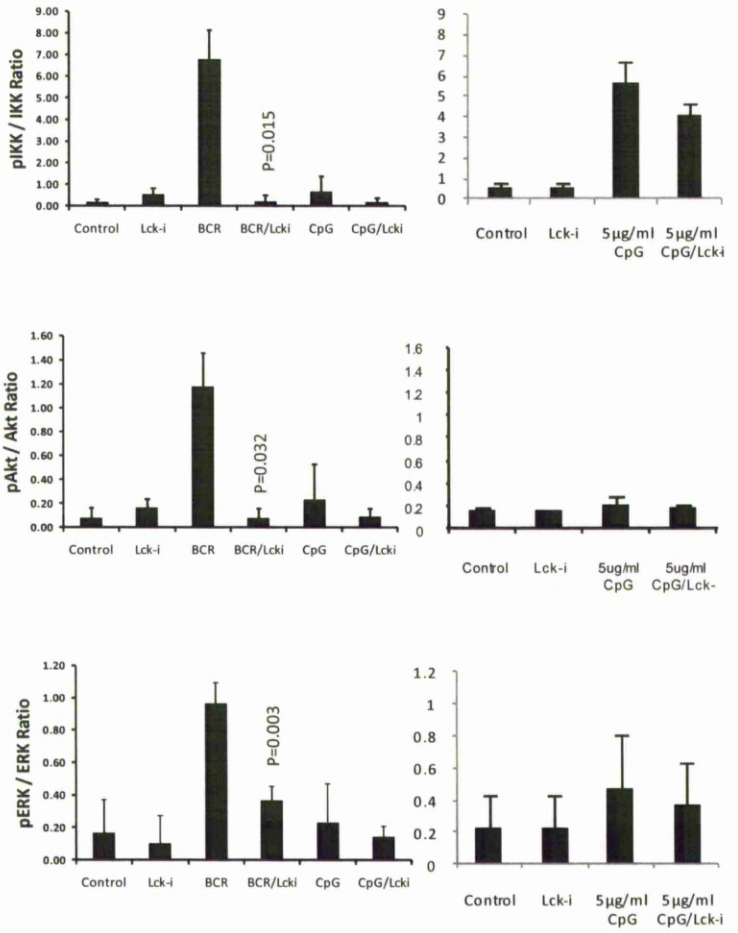


Figure 4.4.B. Phosphorylation of IKK, Akt or ERK by BCR crosslinking, 0.5µg/ml or 5µg/ml CpG stimulation as assesed by densitometry. The phosphorylated to non-phosphorylated protein ratio (mean +/- standard deviation) of three experiments is displayed.

activation of Akt and IKK. In contrast, the presence of the Lck inhibitor had largely no effect on CpG-induced activation of IKK and ERK. In this experiment CpG did not induce an increase in Akt phosphorylation. These results suggest that the Lck inhibitor acts within the pathway of BCR-mediated signals leading to the activation of ERK, Akt and IKK, and that this pathway is distinct from the pathway used by TLRs to activate ERK and IKK. Considering our data showing that the Lck inhibitor reduced Lck but not Lyn activity (Figure 3.2), the above data therefore also suggest that Lck may be central to BCR-induced activation of CLL cells.

4.3.2.2 Role of Lck protein expression levels in BCR responsiveness of CLL cells.

That Lck may be central to BCR-stimulated activation of CLL cells implies that Lck expression levels may have an important role to play in the signalling pathway resulting from this stimulation. Thus, CLL cells expressing high levels of Lck should respond to BCR crosslinking with greater increases in ERK, Akt and IKK phosphorylation than do CLL cells with low levels of Lck. However, Figure 4.5 shows that there is no significant difference in the degree of Akt, ERK or IKK phosphorylation that is induced by BCR stimulation between CLL cases with high levels of Lck and those with low levels of this protein. This suggests that the contribution of Lck to BCR signalling in CLL cells may be tempered by additional other factors.

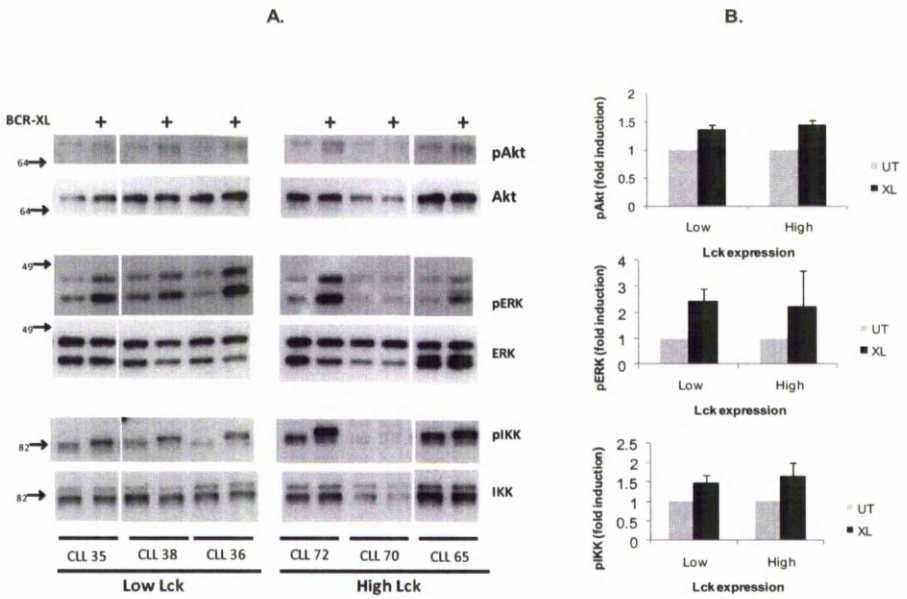


Figure 4.5. Phosphorylation of Akt, ERK and IKK in response to BCR crosslinking in three low versus three high Lck expressing clones. $2x10^6$/ml cells were stimulated with/without 10µg/ml final concentration of F(ab)2 anti-IgM for 15 minutes. 10µg total protein was subjected to Western blotting for phosphorylated or total Akt, ERK or IKK (**A**). Results are representative of two experiments. **B**; representation of the fold induction in phosphorylation in the low versus high Lck expressing clones as determined by densitometry normalised to the amount of total, non-phosphorylated, protein (mean +/- standard deviation).

4.3.2.3 BCR stimulation of CLL cells induces ZAP70 phosphorylation by Lck.

The effect of Lck on activation of signalling components more proximal to the BCR and upstream of those investigated above was studied next. Antibodies that detect phosphorylation of activating tyrosine residues of Btk, Syk and ZAP70 were utilised in CLL clones stimulated with anti-IgM in the presence of the Lck inhibitor. Tyrosine phosphorylation of Y^{551} within Btk primes this protein for full activation, and is mediated by SFKs when Btk translocates to the membrane of cells undergoing activation [215]. Tyrosine phosphorylation of Syk on Y^{323} is mediated by SFKs, but is involved in downregulating Syk activity. In contrast, Y^{525} in Syk is a site of autophosphorylation, phosphorylation on this site is generally seen as a marker of kinase activation [216]. Phosphorylation of Y^{319} within ZAP70 can be catalysed by Lck, and is a marker of activation for this enzyme .

Figure 4.6 shows the results of this analysis. BCR crosslinking led to an increase in ZAP70 phosphorylation in all 3 CLL cases used, and the presence of the Lck inhibitor blocked this increase. However, BCR crosslinking did not induce increases in the levels of either pY^{551}-Btk or pY^{525}-Syk. That these antibodies recognised their epitopes is shown in the last two lanes on the right. Here, the lysates of CLL cells cultured with irradiated fibroblasts prior to BCR crosslinking were probed with the phospho-specific antibodies to Btk, Syk and ZAP70. Thus, these cells showed constitutive activation of Btk and ZAP70 regardless of BCR

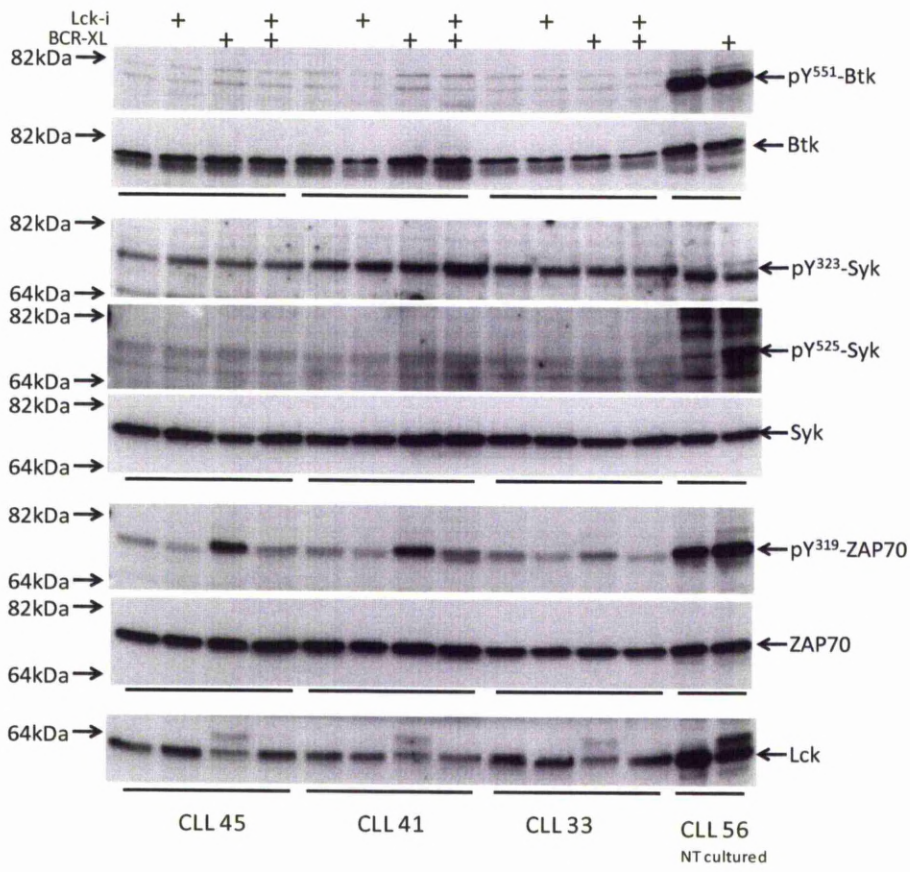


Figure 4.6. Phosphorylation of tyrosine 319 of ZAP70 in response to BCR crosslinking is Lck dependent. Three CLL clones were stimulated for 15 minutes with 10μg/ml F(ab')$_2$ anti-IgM (BCR-XL) in the presence or absence of 1μM Lck inhibitor (Lck-i). A fourth CLL clone was cultured in the presence of non-transfected (NT cultured) fibroblasts with or without 10μg/ml F(ab')$_2$ anti-IgM as a positive control for phosphorylation of Btk, Syk and ZAP70. Results are representative of two experiments.

engagement, whereas the induction of pY^{525}-Syk required BCR stimulation. Interestingly, there appeared to be detectable levels of pY^{323}-Syk present in the CLL cell lysates from all cases used, including the CLL cells cultured with the irradiated fibroblasts, and BCR crosslinking did not seem to affect the degree of phosphorylation in any case. The lysates used in Figure 4.6 are the same as those used to analyse pERK, pAkt and pIKK in Figure 4.4, thus, the fact that pY^{551}-Btk and pY^{525}-Syk are not observed does not indicate that signals from the BCR were not being transmitted. Taken together, the above results suggest that Lck is able to phosphorylate ZAP70 in CLL cells, and that this may be responsible for the downstream signals to the ERK, PI3K and NFκB pathways.

4.4. Discussion

In this chapter I investigated the potential role of Lck in the induction of signalling in CLL cells undergoing BCR engagement. First, I investigated a potential role for Lck in conjunction with CD5 on the basis that this association may downregulate BCR signals. I found that crosslinking CD5 on CLL cells induced an upregulation of CD25 expression, and that was dependent on active Lck. However, further investigation of Lck association with CD5 was not possible. Second, I investigated a potential role for Lck in BCR signalling. Here I found that Lck activity was important for BCR-

induced activation of ERK, Akt, and IKK, and that this might be mediated by the ability of Lck to phosphorylate ZAP70.

I initially investigated a possible role for Lck in CLL cells by examining the potential of these cells to undergo apoptosis in response to CD5 crosslinking. This phenomenon in CLL cells has been previously reported, however, I was unable to repeat these experiments. This was not because the CD5 antibody was without effect, because my experiments showed that treatment of CLL cells with this antibody led to increased CD25 expression. The lack of response was also not due to the CLL subtype used because I specifically chose CLL cell clones whose phenotype matched that which was reported to undergo apoptosis in response to CD5 crosslinking in another study [212]. Other studies have implicated CD5 in providing pro-survival signals to CLL cells [166, 217], however, my results showed that CD5 crosslinking had no significant effect on CLL cell viability. Thus, from my experiments no definitive conclusion can be drawn about the function of Lck in CLL cells with respect to the induction of apoptosis or cell survival.

Nevertheless, I did find that crosslinking CD5 on CLL cells upregulated the expression of CD25, and that this response was dependent on Lck. This result confirms previous work by others [213], and extends this work to show that CD5 on CLL cells operates in a similar way as it does on T cells.

The Lck dependence of CD25 expression by CD5 crosslinking also suggests that Lck may be in association with CD5 in CLL cells. That CD5 may be a target for Lck-mediated phosphorylation in CLL cells is supported by studies demonstrating that this is the case in T cells [164, 210, 218], and by implication in studies of B cells where B-1 cells and anergic B cells have been shown to express both CD5 and Lck [219, 220]. In CLL cells, CD5 is reported to be constitutively phosphorylated [166], and this is thought to regulate the expression of a specific set of genes important for the survival of these cells. In my experiments I probed immunoprecipitated CD5 for the presence of phospho-tyrosine residues. I did not observe CD5 phosphorylation in CLL cells under any condition, however, CD5 phosphorylation could be induced in Jurkat T cells following treatment with the tyrosine phosphatase inhibitor mpV(pic). The best result was observed when I used lysis buffers containing only Triton X-100, presumably because this preserved the secondary and tertiary structures of CD5 that are necessary for efficient immunoprecipitation with the antibody I used. The reasons for my failure to observe CD5 phosphorylation in CLL cells could be due to a failure of CD5 to immunoprecipitate from Triton X-100 solubilised CLL cells; CD5 has been demonstrated to associate with a Triton X-100 insoluble fraction of T cells that have been stimulated with CD5 antibodies [221]. Whatever the reason, this lack of a response in CLL cells meant that I was unable to

investigate the effect of the Lck inhibitor and assess the role of Lck in this process.

I next investigated a potential role for Lck in BCR signalling. The work in this chapter shows that Lck contributes to signalling in response to BCR engagement. I found that BCR-induced phosphorylation / activation of ERK, Akt and IKK in CLL cells was inhibited by the Lck inhibitor. Activation of the signalling pathways leading to phosphorylation of ERK (the MAPK pathway), and, in particular, of Akt (the PI3K pathway) and IKK (the NFκB pathway) is central to the pro-survival effects provided by BCR crosslinking on CLL cells [222-224]. Thus, BCR-mediated activation of Lck may be critical for the induction of CLL cell survival.

The paradigm of BCR-induced signalling in normal B cells involves the activation of Lyn as one of the most proximal events following antigen engagement of the receptor [68]. This role for Lyn in BCR signalling is also accepted with respect to CLL cells because Lyn is overexpressed in CLL cells [132], and because crosslinking the BCR on CLL cells induces translocation of this receptor into lipid raft domains where Lyn is also present [161, 225]. Lyn is reported to be constitutively active in CLL cells [132], and the data I present in Chapter 3 showing that Lyn can be immunoprecipitated from lysates of resting CLL cells using antibodies directed against active Src confirms this result. Thus, my observation that

the Lck inhibitor has no effect on Lyn activity in CLL cells, even when it is used at very high concentrations, indicates that the role of Lck in CLL cells responding to BCR engagement may be more important than previously thought and require a change in paradigm. In this new model of BCR signalling, it is Lck which mediates the membrane proximal events following antigen binding to receptor on CLL cells.

Proof of this role for Lck in BCR signalling could be provided by knockdown of this protein in CLL cells using siRNA. However, the data presented in Chapter 2 of this thesis shows that Lck protein levels are not affected by siRNA targeting Lck mRNA. In the absence of the ability to employ siRNA to reduce Lck expression in CLL cells, I resorted to using cases with different levels of Lck expression. I found that there was no difference in BCR responsiveness, in terms of the induction of pERK, pAkt and pIKK, between CLL cell clones expressing high levels of Lck and those expressing low levels of this protein. This was also true with respect to the induction of CLL cell survival following BCR stimulation. Thus, the role of Lck in BCR signalling in CLL cells, although important, is not governed by a simple relationship between expression level and response. The data presented in this thesis support this notion and show that Lck expression in CLL cells does not correlate with markers of poor disease prognosis such as IgVH mutation and ZAP70 expression. This may be important because the cohort of CLL cases used in this study show, in

agreement with published data [63, 65, 226], good correlation between ZAP70 expression and IgVH mutation. That is, the malignant cells from unmutated CLL cases have high levels of ZAP70 expression whereas those from mutated CLL cases have low levels of ZAP70. Considering the established role of the BCR for CLL clonal expansion, that unmutated IgVH genes are important because of associated broad antigen specificity of the BCR [48] and that the presence of ZAP70 is an important enhancer of BCR signalling [168], it is easy to see why ZAP70 expression and IgVH gene mutation are important indicators of poor prognosis in CLL. That Lck expression levels do not correlate with either of these markers but is nevertheless still important for the transmission of BCR signals suggests that additional factors are important with respect to more distal events (to Lck) in the BCR signalling pathway.

One of these additional factors could be ZAP70 expression. I show in this chapter that Lck is responsible for phosphorylation of ZAP70 on tyrosine 319 (figure 4.6). In T cells this tyrosine residue is situated within a binding site for the SH2 domain of Lck [227], and is required for ZAP70 activation and propagation of the downstream signalling cascade [119]. Phosphorylation of Y^{319} in ZAP70 was originally thought to be mediated by autophosphorylation, and that the contribution of Lck was to facilitate ZAP70 activation by phosphorylating the ITAM motifs within the components of the TCR that attracted ZAP70 to bind and activate this

protein. However, it has now been shown that Y^{319} within ZAP70 is a substrate of Lck, and that its phosphorylation does not affect the enzymatic activity of ZAP70 but instead influences its association with other intracellular transducers such as Lck [227] and PLCγ1 [228]. The structure of ZAP70, including the Y319 phosphorylation site, is shown in figure 4.7.

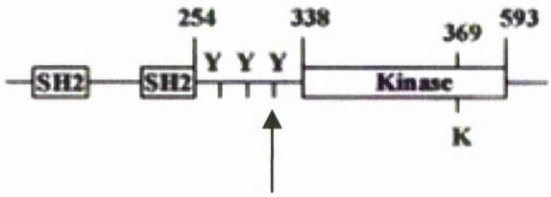


Figure 4.7 The structure of ZAP70. Adapted from [229].

It has been suggested that the role ZAP70 plays in CLL pathogenesis is independent of its kinase activity and relies on its role as an adapter protein [168, 230]. This was concluded from work on ZAP70 negative CLL which were transfected with ZAP70 containing a mutation of K^{369} to alanine which results in a form of ZAP70 that cannot be activated. The result in figure 4.6 supports the role of Lck in BCR crosslinking-induced signalling as Lck can phosphorylate Y^{319} and lead to an increase of ZAP70 binding to signalling components that could contribute to its pathogenesis in CLL as an adapter protein.

The fact that the Lck inhibitor affects CLL clones with unmutated IgVH genes to a greater extent than those with mutated sequences supports the proposal that Lck contributes to CLL cell survival by phosphorylating ZAP70, which is expressed to a much higher degree in unmutated than in mutated cells. However, the Lck inhibitor also affects survival of clones with mutated IgVH sequences. This suggests that Lck has additional roles in CLL cells other than just phosphorylation of ZAP-70 Y^{319}, as mutated cases contain low to absent levels of ZAP70 protein.

The role of Syk (p^{72}Syk) kinase activity has been suggested to be more important for CLL pathogenesis than the kinase activity of ZAP70. Syk has an analogous role in B cells to the role of ZAP70 in T cells. Syk is recruited to phosphorylated ITAMs of CD79a and CD79b of the BCR complex leading to activation of Syk and increased downstream signalling [68]. The presence of ZAP70 and its role as an adapter protein is thought to contribute to increased activation of Syk which has a 100-fold greater kinase activity in CLL cells *in vitro* than does ZAP70 [231, 232]. Therefore future studies of the role of Lck in CLL antigen receptor signalling would also have to include the potential role of Lck in the activation of Syk. Figure 4.6 suggests that Lck is not involved in the phosphorylation of Y^{323} or Y^{525}. Y^{323} in Syk can be phosphorylated either by Syk itself, or by SFKs, including Lyn [216]. Phosphorylation of Syk at this site is associated with downregulation of Syk activity and expression

though association with Casitas B-lineage lymphoma (Cbl) family proteins, as well as with activation of PI3K through association with the p85α subunit. In my experiments, presence of the Lck inhibitor did not affect pY^{323}-Syk levels in CLL cells, suggesting that Lck is not responsible for phosphorylation of Syk at this site. Y^{525} in Syk is located within the activation loop of the kinase, and is a site of autophosphorylation that is thought to be important for initial activation of the kinase [216]. In my experiments BCR stimulation did not result in an increase in pY^{525}-Syk levels. The reasons for this are not clear, but are not due to a failure of the phospho-specific antibody to recognise pY^{525}-Syk since this antibody did recognise this epitope under appropriate conditions of BCR activation in the positive control lanes. Nevertheless, phosphorylation of Syk at Y^{525} did not seem to be necessary for distal signalling to take place because BCR crosslinking activated the MAPK, PI3K and NFκB pathways within these cells.

Taken together, the results of this Chapter suggest an important role for Lck in transmitting BCR signals in CLL cells. However, the exact nature of this role remains to be further clarified.

Chapter 5

General discussion and future work

The aim of the work described in this Thesis was to gain some insight into the expression and function of the protein tyrosine kinase Lck in the malignant cells of CLL. So far this enzyme has been extensively studied only in T lymphocytes where it plays a key role in the cell response to various stimuli that regulate selection, clonal expansion and function of these cells.

Although initially thought to be a T cell-specific protein, Lck was later found in a number of other normal and malignant cells, including normal B-1 cells and CLL cells [4]. Since in normal B cells Lyn plays an equivalent role to that of Lck in T cells, this raised the question of whether SFKs expressed in CLL cells could have important specific roles or be largely redundant. The published studies carried out to answer this question show that these highly homologous enzymes are likely to have both specific and shared roles, but such roles of Lck in CLL cells have yet to be described.

Other questions of particular relevance for normal B cells and malignant CLL cells are those related to the regulation of expression and function of Lck in different subsets of normal B cells which are at different stages of maturation and activation as well as in cells from prognostically distinct sub-groups of CLL.

For the purposes of the project described in this Thesis, it was assumed that the expression of Lck is another phenotypic marker of CLL cells supporting their origin from B-1 cells that have been positively selected by a restricted set of T-independent auto antigens but can also react with structurally similar microbial products. However in contrast to normal B-1 cells that are responsible for natural antibody production, CLL cells are arrested at the pre-plasma cell stage of differentiation and remain chronically stimulated by cognate antigens by being unable to produce significant levels of antibodies that could neutralize and initiate clearance of these antigens.

In the Chapter concerned with the regulation of Lck expression, attention was paid to all three stages of this regulation, namely transcription, translation and post-translational modification. During lymphocyte development Lck expression is regulated at the level of transcription by signals generated through cell stimulation by antigens and/or LPS [3]. Antigen receptor cross-linking on normal mature lymphocytes in the

presence of IL-2 also results in an increase in Lck mRNA [113], but these mature cells activate different promoters from that activated in immature cells [3]. Moreover, the promoters responsible for Lck mRNA transcription in malignant cells are again different from those utilized by their normal counterparts. It was therefore decided to analyse all transcripts that could possibly be generated in CLL cells through activation of both proximal (type I) and distal (type II) promoters with the aim that this could provide insights into whether Lck expression in these cells reflects the state of their maturation or activation, or it is only a consequence of their malignant transformation.

CLL cells contained both Type I and Type II transcripts, the Type II transcript - which is in normal mature lymphocytes associated with antigen receptor signalling - was the dominant product of Lck transcription in these malignant cells. The malignant nature of these cells was further underscored by expression of variable levels of type Ia and Ib transcripts which are normally associated with cells undergoing malignant transformation. This analysis of Lck mRNA transcripts therefore supports the generally accepted notion that CLL cells are mature, antigen-activated malignant cells.

The study of Lck transcription levels in different CLL clones by qRT-PCR showed that all cells expressed a high level of Lck mRNA in comparison to normal B cells and the malignant cells of HCL. The levels of Lck

mRNA in the different CLL clones studied were similar to the levels in normal T cells.

At the onset of the work described in this thesis the presence of Lck in CLL cells was still questioned in some publications. However, the work in this department clearly showed that in the limited number of clones studied, all contained functional Lck protein which was detectable by Western blotting and had clear protein tyrosine activity in an *in vitro* assay [190]. Nevertheless at that stage it was still not clear whether Lck is expressed in all CLL cells belonging to different subgroups of the disease, or only in some cells as demonstrated in mice through comparison of B-1 cells from peritoneum and spleen. In addition, it has been already shown that Lck expression can depend on the stage of cell maturation as well as on the degree of cell activation, but whether different CLL cells differ in these regards was also still unknown.

The study presented here demonstrates that CLL cells, belonging to by now well characterised disease subgroups, all contain variable amounts of Lck ranging from barely detectable to as high as in T cells and that this Lck does not originate from contaminating T cells. In 42 CLL clones where the malignant cells were purified by removing contaminating T cells, monocytes/macrophages and NK cells the amount of Lck varied greatly

but did not correlate with any disease prognosis markers such as IgVH gene mutation or ZAP70 expression.

When the levels of Lck mRNA were compared to the level of Lck protein present in each case, it was shown that there was no correlation. Thus, despite an almost 10-fold difference in protein expression, Lck mRNA levels were relatively constant. This indicates that the level of Lck protein present in each case is the result of regulation at the post-transcriptional level. It also indicates that the half life of the protein is very long, and suggests a reason why siRNA targeting Lck mRNA did not result in a reduction of protein levels.

In normal B cells, Lck expression can be induced by BCR crosslinking and IL-2 [113] but these stimuli failed to up-regulate Lck expression in CLL cells. Thus the pathway that in normal B cells leads to increased Lck expression in response to BCR stimulation and IL-2 seems to be defective in CLL cells, although other results in this thesis show that BCR stimulation effectively activates Lck. However since Lck expression could be potentially induced by a number of other stimuli, and since all CLL cells are thought to be arrested at a similar level of maturation, it still seems likely that Lck expression varies with the degree of cell activation by a mechanism that remains to be identified.

The post-translational regulation of Lck protein levels in T cells is well described. In response to TCR engagement or PKC activators Lck is activated and then serine phosphorylated, which causes a shift on SDS-PAGE analysis. I show in this thesis that Lck in CLL cells undergoes similar post-translational modifications. BCR crosslinking and PMA/Bryostatin stimulation result in a shift in molecular weight of Lck corresponding to the appearance of the p60 band. Moreover, I also show that this is due to phosphorylation of Lck, most likely on the S^{42} and S^{59} residues that are targeted by PKC and MAPKs.

In T cells, phosphorylated Lck is then targeted for proteasomal degradation by polyubiquitinylation [96]. The results presented in this thesis suggest that this level of post-translational regulation of Lck protein levels in CLL cells may differ from the process in T cells. There was some evidence of ubiquitinylation of Lck in response to CLL cell activation by BCR engagement or bryostatin stimulation. However, the analysis of the fate of the p60 band of Lck was complicated by the sensitivity of CLL cells to the cytotoxic effects of the proteasomal inhibitor lactacystin and by the transcriptional inhibitors flavopiridol and actinomycin D within the 12 – 24h culture times of the experiments I performed. Nevertheless, because flavopiridol (at a 4h time point where CLL cell viability was unaffected) caused a quantitative reduction in Mcl1 levels while Lck was unaffected, I can say that Lck protein levels are not rapidly turned over in the cell, even

when the cells have been stimulated with bryostatin. Here, the data support a notion that phosphorylated Lck in CLL cells is turned over at a far slower rate than it would in T cells, and that the reappearance of the p56 band in CLL cells on prolonged culture is likely to be the result of dephosphorylation.

The regular presence of Lck in CLL cells posed the question of the functional role of this enzyme and whether it contributes to the pathogenesis of the disease. Since survival of CLL cells undoubtedly contributes to their clonal expansion and can be measured *in vitro*, the next step was to examine the effect of Lck inhibition on the *in vitro* survival of unstimulated and variously stimulated CLL cells. Attempts to use siRNA to block Lck production in CLL cells did not result in the desired lowering of Lck protein. Therefore it was necessary to use a pharmacological inhibitor of Lck in all experiments designed to determine its effects on cell survival, the signalling pathways involved and the underlying mechanism responsible for the change in the balance between cell survival and cell death caused by these signals. Firstly, I confirmed that the Lck inhibitor acted to inhibit the activity of Lck and not the activity of Lyn, as this latter protein is highly expressed and constitutively active in CLL cells [132]. To determine whether Lck is involved in preservation of cell viability, unstimulated CLL cells from 4 different patients were incubated with a range of concentrations of the Lck inhibitor for 48 hours and their viability

then determined using a DiOC6/PI apoptosis assay. HCs do not contain demonstrable amounts of Lck protein, and were therefore used as negative controls. The results of this experiment showed that the viability of all CLL cell clones tested, but not of HCs, was variably decreased by the Lck inhibitor, with 50% of this decrease occurring at around 1μM in the most sensitive cases.

When the number of different CLL cells tested was increased to include 27 IgVH mutated and 29 unmutated clones it was found that the Lck inhibitor at 1μM concentration had a considerably larger pro-apoptotic effect on unmutated than mutated cells while HC viability was not affected at all. A previous study from our department has shown that unmutated cells more readily respond to BCR cross-linking by an increase in cell viability accompanied by ERK activation [190]. Therefore these observations together with the above effects of the Lck inhibitor suggest that Lck may be participating in the preservation of cell viability by being a component of a signalling pathway that is activated by cognate antigens and also includes activation of ERK. However since many alternative stimuli and signalling pathways that protect CLL cells from apoptosis were proposed in the literature, the viability of CLL cells and the effect of the Lck inhibitor were also determined in cells stimulated not only by anti-IgM but also CD40L or CpG oligonucleotide (ODN 2006).

The results of these experiments showed that BCR crosslinking, CD40 engagement and TLR signalling induced increased CLL cell survival, and that the presence of the Lck inhibitor blocked this increase regardless of the stimulus. This suggests that the role of Lck in CLL cell cytoprotection is not restricted to a single pathway, and that Lck may be involved in the regulation of anti-apoptosis proteins. I therefore next investigated the effect of the Lck inhibitor on the expression of Bcl2 family and IAP family proteins in CLL cells. I found that the presence of this agent in CLL cell cultures reduced the expression of XIAP and cIAP2. This reduction of expression in XIAP and cIAP2 was not rescued by the presence of zVAD, suggesting that Lck inhibitor did not promote caspase cleavage of these proteins, but was likely involved in the maintenance of high expression levels. I also found that the presence of Lck inhibitor in CLL cell cultures resulted in an increase in Bim protein levels. Thus, the combination of increased pro-apototic Bim and decreased anti-apoptotic XIAP likely stimulated mitochondrial depolarisation resulting in caspase activation and cleavage of anti-apototic Mcl1 and Bcl-xL to further facilitate the process of apoptosis.

In order to determine which pathway(s) are involved in Lck-dependent cytoprotection, $F(ab')_2$ anti-IgM or CpG oligonucleotide stimulated CLL cells were examined for the presence of phosphorylated (activated) ERK, Akt and IKKα/β, in the presence and absence of the Lck inhibitor.

I found that the presence of the Lck inhibitor profoundly blocked BCR-mediated activation of ERK, Akt and IKK, whereas its effects on CpG stimulation were negligible. This result matches the survival data because the Lck inhibitor had a greater effect on blocking the pro-survival effects of BCR crosslinking than it did on those of TLR stimulation. These results therefore strongly indicate that Lck may be central to BCR signalling in CLL cells. This role of Lck in BCR signalling is supported by my experiments showing that BCR-induced ZAP70 phosphorylation is blocked by the presence of the Lck inhibitor. ZAP70 is an important substrate of Lck in T cells. The exact role of Lck in this process requires further studies. Because *in vivo,* CLL cells are exposed to a large combination of different stimuli and their response to these stimuli may differ in phenotypically different subgroups of cells, it was necessary to concentrate on one single aspect of Lck involvement in the biology of these cells and leave the rest for the future work. The aspect chosen as a natural follow up of the work already carried out in this department and the work discussed above was the role of Lck in ERK activation and the possible link of this activation to the mechanism of cytoprotection.

In conclusion, this work was an attempt to explore still controversial questions of the tissue specificity and redundancy of different Src family kinases using CLL cells as a suitable model cell for such exploration. Differences between normal B cells and CLL cells belonging to different

subgroups with regard to the expression and function of antigen receptor suggests a possibility of the involvement of an alternative Src family kinase in the cellular processes of CLL cells that in normal B cells involve Lyn. This work is the first to demonstrate a cytoprotective mechanism in CLL cells that could specifically involve Lck and might be relevant for survival and clonal expansion of these cells. Like Lyn in normal B cells, Lck in CLL cells seems to be involved in several cytoprotective pathways that are activated through BCR stimulation. With regard to the importance of these studies for the pathogenesis and treatment of CLL, the most relevant future studies are likely to be those aimed at clarification of whether or not Lck cytoprotection is equally relevant for all different subgroups of the disease.

APPENDIX

Table 1.2 Clinical details of CLL cases studied.

Case no (a)	WBC (b)	CD38 (c)	Binet (d)	Rai (e)	VH% (f)	Gene usage (g)	Class (h)
1	167	4	C	III	-	-	-
2	98	82	A	I	1.02	3-07/DP-54	M
3	-	-	-	-	0.34	3-07/DP-54	M
4	33	87	C	IV	0	1-69/DP-10	M
5	310	16	C	IV	0.42	5-51/DP-73	M
6	57	43	C	IV	3.38	3-23/DP-47	M
7	57	43	C	IV	3.38	3-23/DP-47	M
8	129	43	C	IV	3.38	3-23/DP-47	M
9	84	43	C	IV	3.38	3-23/DP-47	M
10	48	10	A	I	0	1-69/DP-10	M
11	46	14	-	-	4.67	4-30.1/DP-65	M
12	80	64	C	IV	0	3-30/DP-46	G
13	163	14	C	IV	0	3-21/DP-77	M
14	21	14	B	I	1.09	3-66/8-1B+	M
15	57	13	A	0	2.8	3-21/DP-77	M
16	122	-	B	I	0	1-69/DP-10	M
17	427	98	B	II	0	1-18/DP-14	M
18	192	-	C	III	0	5-51/DP-73	M
19	134	-	B	0	0.35	1.46/DP-7	M
20	125	-	-	-	0	1-69/DP-10	-
21	129	4	B	II	8.06	4-34/DP-63	G
22	155	-	-	-	5.37	4-34/DP-63	G
23	330	-	C	iV	2.46	3-30.3/DP-46	M
24	33	-	A	0	3.7	3-07/DP-54	M
25	128	-	C	III	10.47	3-30/DP-49	M
26	18	7	A	0	3.64	3-73/YAC-9	M
27	132	53	-	-	0	4-b*01	M
28	170	-	C	II	2.46	3-30.3/DP-46	M
29	158	-	C	III	8.93	4-34/DP-63	M
30	240	97	-	-	0	5-51*01	A
31	285	98	B	II	0	1-18/DP-14	M
32	19	-	B	I	0	VH3-30/DP-49	M
33	382	-	-	-	0	DP8+	M
34	105	27	B	I	0	1-24*01	M
35	142	-	-	-	0.4	3-48/	M
36	118.5	-	-	-	3.6	3-73/	M
37	242	-	-	-	12.3	3-53/	G
38	126.5	-	C	-	0.0	3-09/	M
39	184	-	-	-	0.0	3-30/	M
40	183	-	-	-	0.3	2-70/	M
41	23.3	-	A	-	1.35	3-48/	M
42	412	-	-	-	0	DP8	M
43	86.8	-	B	-	1.09	3-66/	M

Case no (a)	WBC (b)	CD38 (c)	Binet (d)	Rai (e)	VH% (f)	Gene usage (g)	Class (h)
44	49.3	0	A	-	-	-	-
45	215	low	A	0	4.47	4-43/	M
46	128	-	-	-	3.2	3-48/	M
47	116	-	A	-	6.1	3-23/	G
48	24.7	-	A	0	8.5	3-13/	M
49	66.4	low	C	-	11.11	3-48/	M
50	124.7	-	-	-	0.0	1-69/	M
51	147	60	A	-	0	4-34/	M
52	124	-	B	-	6.12	V3-49	G
53	110.7	-	-	-	8.9	4-34/	G
54	187	-	B	-	0.0	4-31/	M
55	182	-	B	-	6.45	1-69/	M
56	266	-	-	-	0.3	3-49/	M
57	-	25	-	-	0.35	1.46	M
58	210	-	B	-	8.6	3-09/	M
59	165	-	-	-	4.6	4-34/	M
60	127	-	-	-	0.0	1-69/	M
61	165	-	A	-	4.56	4-34/	M
62	71.5	-	-	-	-	-	-
63	165	-	-	-	4.6	4-34/	M
64	54	-	-	-	6.12	3-72/	M
65	196	-	-	-	3.1	3-30/	M
66	127	-	B	-	-	-	-
67	150	-	-	-	5.2	3-48/	M
68		-	-	-	-	-	-
69	98.8	-	A	-	4.4	3-07/	M
70	175	-	-	-	6.6	1-69/	M
71	174	-	A	-	1.75	4-59/	M
72	105	-	-	-	1.8	4-59/	M
73	77	-	B	II	1.7	4-61/	M
74	105	-	-	-	2.6	3-48/	M
75	152	-	-	-	0	-	-

a. Case number used in this thesis
b. White blood cell count ($x10^9/L$)
c. % CD38 positive cells
d. Binet clinical staging
e. Rai clinical staging
f. IgVH gene % deviation from germ line sequence
g. IgVH gene segment usage
h. Class of immunoglobulin

Case numbers 2/3, 6-9 and 21/22 are subsequent rebleeds of the same patient.

Lck mRNA sequences

NM_001042771.1 LCK, transcript variant 1, mRNA

GTGTGAATTTACTTGTAGCCTGAGGGCTCAGAGGGAGCACCGGTTTGGAGCT
GGGACCCCCTATTTTAGCTTTTCTGTGGCTGGTGAATGGGGATCCCAGGATCT
CACAATCTCAGGGACCATGGGCTGTGGCTGCAGCTCACACCCGGAAGATGAC
TGGATGGAAAACATCGATGTGTGTGAGAACTGCCATTATCCCATAGTCCCACT
GGATGGCAAGGGCACGCTGCTCATCCGAAATGGCTCTGAGGTGGGGACCCA
CTGGTTACCTACGAAGGCTCCAATCCGCCGGCTTCCCCACTGCAAGACAACCT
GGTTATCGCTCTGCACAGCTATGAGCCCTCTCACGACGGAGATCTGGGCTTTG
AGAAGGGGGAACAGCTCCGCATCCTGGAGCAGAGCGGCGAGTGGTGGAAGG
CGCAGTCCCTGACCACGGGCCAGGAAGGCTTCATCCCCTTCAATTTTGTGGCC
AAAGCGAACAGCCTGGAGCCCGAACCCTGGTTCTTCAAGAACCTGAGCCGCA
AGGACGCGGAGCGGCAGCTCCTGGCGCCCGGGAACACTCACGGCTCCTTCCT
CATCCGGGAGAGCGAGAGCACCGCGGGATCGTTTTCACTGTCGGTCCGGGAC
TTCGACCAGAACCAGGGAGAGGTGGTGAAACATTACAAGATCCGTAATCTGG
ACAACGGTGGCTTCTACATCTCCCCTCGAATCACTTTTCCCGGCCTGCATGAA
CTGGTCCGCCATTACACCAATGCTTCAGATGGGCTGTGCACACGGTTGAGCCG
CCCCTGCCAGACCCAGAAGCCCCAGAAGCCGTGGTGGGAGGACGAGTGGGA
GGTTCCCAGGGAGACGCTGAAGCTGGTGGAGCGGCTGGGGGCTGGACAGTTC
GGGGAGGTGTGGATGGGGTACTACAACGGGCACACGAAGGTGGCGGTGAAG
AGCCTGAAGCAGGGCAGCATGTCCCCGGACGCCTTCCTGGCCGAGGCCAACC
TCATGAAGCAGCTGCAACACCAGCGGCTGGTTCGGCTCTACGCTGTGGTCAC
CCAGGAGCCCATCTACATCATCACTGAATACATGGAGAATGGGAGTCTAGTG
GATTTTCTCAAGACCCCTTCAGGCATCAAGTTGACCATCAACAAACTCCTGGA
CATGGCAGCCCAAATTGCAGAAGGCATGGCATTCATTGAAGAGCGGAATTAT
ATTCATCGTGACCTTCGGGCTGCCAACATTCTGGTGTCTGACACCCTGAGCTG
CAAGATTGCAGACTTTGGCCTAGCACGCCTCATTGAGGACAACGAGTACACA
GCCAGGGAGGGGGCCAAGTTTCCCATTAAGTGGACAGCGCCAGAAGCCATTA
ACTACGGGACATTCACCATCAAGTCAGATGTGTGGTCTTTTGGGATCCTGCTG
ACGGAAATTGTCACCCACGGCCGCATCCCTTACCCAGGGATGACCAACCCGG
AGGTGATTCAGAACCTGGAGCGAGGCTACCGCATGGTGCGCCCTGACAACTG
TCCAGAGGAGCTGTACCAACTCATGAGGCTGTGCTGGAAGGAGCGCCCAGAG
GACCGGCCCACCTTTGACTACCTGCGCAGTGTGCTGGAGGACTTCTTCACGGC
CACAGAGGGCCAGTACCAGCCTCAGCCTTGAGAGGCCTTGAGAGGCCCTGGG
GTTCTCCCCCTTTCTCTCCAGCCTGACTTGGGGAGATGGAGTTCTTGTGCCAT
AGTCACATGGCCTATGCACATATGGACTCTGCACATGAATCCCACCCACATGT
GACACATATGCACCTTGTGTCTGTACACGTGTCCTGTAGTTGCGTGGACTCTG
CACATGTCTTGTACATGTGTAGCCTGTGCATGTATGTCTTGGACACTGTACAA
GGTACCCCTTTCTGGCTCTCCCATTTCCTGAGACCACAGAGAGAGGGGAGAA
GCCTGGGATTGACAGAAGCTTCTGCCCACCTACTTTTCTTTCCTCAGATCATC
CAGAAGTTCCTCAAGGGCCAGGACTTTATCTAATACCTCTGTGTGCTCCTCCT
TGGTGCCTGGCCTGGCACACATCAGGAGTTCAATAAATGTCTGTTGATGACTG
TTGTAAAAAAAAAAAAAAAAAA

NM_005356.3 LCK, transcript variant 2, mRNA

AGTCAGGGTGGGACGTGGGCGCGGGGAGACAGGTGGTGGCTACGACGGCGA
AGGGAGCTGAGACTGTCCAGGCAGCCAGGTTAGGCCAGGAGGACCATGTGA
ATGGGGCCAGAGGGCTCCCGGGCTGGGCAGGGACCATGGGCTGTGGCTGCAG
CTCACACCCGGAAGATGACTGGATGGAAAACATCGATGTGTGTGAGAACTGC
CATTATCCCATAGTCCCACTGGATGGCAAGGGCACGCTGCTCATCCGAAATG
GCTCTGAGGTGGGGACCCACTGGTTACCTACGAAGGCTCCAATCCGCCGGC
TTCCCCACTGCAAGACAACCTGGTTATCGCTCTGCACAGCTATGAGCCCTCTC
ACGACGGAGATCTGGGCTTTGAGAAGGGGGAACAGCTCCGCATCCTGGAGCA

GAGCGGCGAGTGGTGGAAGGCGCAGTCCCTGACCACGGGCCAGGAAGGCTT
CATCCCCTTCAATTTTGTGGCCAAAGCGAACAGCCTGGAGCCCGAACCCTGGT
TCTTCAAGAACCTGAGCCGCAAGGACGCGGAGCGGCAGCTCCTGGCGCCCGG
GAACACTCACGGCTCCTTCCTCATCCGGGAGAGCGAGAGCACCGCGGGATCG
TTTTCACTGTCGGTCCGGGACTTCGACCAGAACCAGGGAGAGGTGGTGAAAC
ATTACAAGATCCGTAATCTGGACAACGGTGGCTTCTACATCTCCCCTCGAATC
ACTTTTCCCGGCCTGCATGAACTGGTCCGCCATTACACCAATGCTTCAGATGG
GCTGTGCACACGGTTGAGCCGCCCCTGCCAGACCCAGAAGCCCCAGAAGCCG
TGGTGGGAGGACGAGTGGGAGGTTCCCAGGGAGACGCTGAAGCTGGTGGAG
CGGCTGGGGGCTGGACAGTTCGGGGAGGTGTGGATGGGGTACTACAACGGGC
ACACGAAGGTGGCGGTGAAGAGCCTGAAGCAGGGCAGCATGTCCCCGGACG
CCTTCCTGGCCGAGGCCAACCTCATGAAGCAGCTGCAACACCAGCGGCTGGT
TCGGCTCTACGCTGTGGTCACCCAGGAGCCCATCTACATCATCACTGAATACA
TGGAGAATGGGAGTCTAGTGGATTTTCTCAAGACCCCTTCAGGCATCAAGTTG
ACCATCAACAAACTCCTGGACATGGCAGCCCAAATTGCAGAAGGCATGGCAT
TCATTGAAGAGCGGAATTATATTCATCGTGACCTTCGGGCTGCCAACATTCTG
GTGTCTGACACCCTGAGCTGCAAGATTGCAGACTTTGGCCTAGCACGCCTCAT
TGAGGACAACGAGTACACAGCCAGGGAGGGGGCCAAGTTTCCCATTAAGTGG
ACAGCGCCAGAAGCCATTAACTACGGGACATTCACCATCAAGTCAGATGTGT
GGTCTTTTGGGATCCTGCTGACGGAAATTGTCACCCACGGCCGCATCCCTTAC
CCAGGGATGACCAACCCGGAGGTGATTCAGAACCTGGAGCGAGGCTACCGCA
TGGTGCGCCCTGACAACTGTCCAGAGGAGCTGTACCAACTCATGAGGCTGTG
CTGGAAGGAGCGCCCAGAGGACCGGCCCACCTTTGACTACCTGCGCAGTGTG
CTGGAGGACTTCTTCACGGCCACAGAGGGCCAGTACCAGCCTCAGCCTTGAG
AGGCCTTGAGAGGCCCTGGGGTTCTCCCCCTTTCTCTCCAGCCTGACTTGGGG
AGATGGAGTTCTTGTGCCATAGTCACATGGCCTATGCACATATGGACTCTGCA
CATGAATCCCACCCACATGTGACACATATGCACCTTGTGTCTGTACACGTGTC
CTGTAGTTGCGTGGACTCTGCACATGTCTTGTACATGTGTAGCCTGTGCATGT
ATGTCTTGGACACTGTACAAGGTACCCCTTTCTGGCTCTCCCATTTCCTGAGA
CCACAGAGAGAGGGGAGAAGCCTGGGATTGACAGAAGCTTCTGCCCACCTAC
TTTTCTTTCCTCAGATCATCCAGAAGTTCCTCAAGGGCCAGGACTTTATCTAA
TACCTCTGTGTGCTCCTCCTTGGTGCCTGGCCTGGCACACATCAGGAGTTCAA
TAAATGTCTGTTGATGACTGTTGTAAAAAAAAAAAAAAAAAAA

RT-PCR forward primers

RT-PCR reverse primer

qRT-PCR forward primer

qRT-PCR reverse primer

Buffers

TBST

Tris (pH 7.4)	150mM
Sodium chloride	50mM
Tween-20	0.1%

Running gel buffer (4x)

Tris (pH 8.8)	1.5M
SDS	0.4%(w/v)

Running gel

Acrylamide	Varied (usually 10%)
4x Running gel buffer	25%
TEMED	0.01%
Ammonium persulphate	0.04%(w/v)

Made up to 100% with dH_2O

Stacking gel buffer

Tris (pH 6.8)	0.5M
SDS	0.4%(w/v)

Stacking gel

Acrylamide	4%
Stacking gel buffer	25%
TEMED	0.1%
Ammonium persulphate	0.08%(w/v)

Made up to 100% with dH_2O.

Transfer buffer

Bought from Geneflow Ltd (Fradley, UK)

Electro buffer

Tris	25mM
Glycine	192mM
SDS	0.1%(w/v)

Western blot stripping buffer

SDS	2%(w/v)
Tris (pH6.7)	62.5mM
β-mercaptoethanol	100mM

Laemelli buffer (2x)

Tris (pH6.8)	125mM
Glycerol	20%

SDS	4%(w/v)
β –mercaptoethanol	10%
Bromophenol blue	0.006%(w/v)

Clear lysis buffer

SDS	1%(w/v)
Glycerol	10%
Tris (pH6.8)	50mM
EDTA	5mM

Phosphate Buffered Saline (PBS)

NaCl	150mM
KCl	3mM
Na_2HPO_4	4.3mM
KH_2PO_4	1.5mM

Purification buffer

PBS

BSA	0.1%(w/v)
EDTA	2mM

Degassed overnight prior to use.

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